

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 September 2002 (12.09.2002)

PCT

(10) International Publication Number  
**WO 02/069947 A2**

(51) International Patent Classification<sup>7</sup>: **A61K 31/00**

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(21) International Application Number: PCT/IB02/02002

(22) International Filing Date: 14 January 2002 (14.01.2002)

(25) Filing Language: English

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(26) Publication Language: English

(30) Priority Data:  
60/261,674 12 January 2001 (12.01.2001) US  
60/261,522 12 January 2001 (12.01.2001) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

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**Published:**

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/069947 A2

(54) Title: METHODS FOR SPECIFICALLY INHIBITING HISTONE DEACETYLASE-4

(57) Abstract: This invention relates to the inhibition of histone deacetylase (HDAC) expression and enzymatic activity. The invention provides methods and reagents for inhibiting HDAC-4 and HDAC-1 by inhibiting expression at the nucleic acid level or inhibiting enzymatic activity at the protein level.

(Case No. MET-002PC)

## BACKGROUND OF THE INVENTION

## Field of the Invention

10 expression and oncology.

### Summary of the Related Art

15 structural organization of chromatin. The nucleosome principally consists of  
(1) the core histones, termed H2A, H2B, H3, and H4, which associate to form a  
protein core particle, and (2) the approximately 146 base pairs of DNA  
wrapped around the histone core particle. The physical interaction between  
the core histone particle and DNA principally occurs through the negatively  
20 charged phosphate groups of the DNA and the basic amino acid moieties of  
the histone proteins. (Csordas, *Biochem. J.*, 286:23-38 (1990)) teaches that  
histones are subject to posttranslational acetylation of their epsilon-amino  
groups of *N*-terminal lysine residues, a reaction that is catalyzed by histone  
acetyl transferase (HAT). The posttranslational acetylation of histones has  
25 both structural and functional, *i.e.*, gene regulatory, consequences.

Acetylation neutralizes the positive charge of the epsilon-amino groups of *N*-terminal lysine residues, thereby influencing the interaction of DNA with the histone core particle of the nucleosome. Thus, histone acetylation and histone deacetylation (HDAC) are thought to impact chromatin structure

and gene regulation. For example, Taunton *et al.*, *Science*, 272:408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent  
5 regions of the genome.

Studies utilizing known HDAC inhibitors have established a link between acetylation and gene expression. Yoshida *et al.*, *Cancer Res.* 47:3688-3691 (1987) discloses that (R)-Trichostatin A (TSA) is a potent inducer of differentiation in murine erythroleukemia cells. Yoshida *et al.*, *J. Biol. Chem.*  
10 265:17174-17179 (1990) teaches that TSA is a potent inhibitor of mammalian HDAC.

Numerous studies have examined the relationship between HDAC and gene expression. Taunton *et al.*, *Science* 272:408-411 (1996), discloses a human HDAC that is related to a yeast transcriptional regulator. Cress *et al.*, *J. Cell.*  
15 *Phys.* 184:1-16 (2000), discloses that, in the context of human cancer, the role of HDAC is as a corepressor of transcription. Ng *et al.*, *TIBS* 25:March (2000), discloses HDAC as a pervasive feature of transcriptional repressor systems. Magnaghi-Jaulin *et al.*, *Prog. Cell Cycle Res.* 4:41-47 (2000), discloses HDAC as a transcriptional co-regulator important for cell cycle progression.

20 The molecular cloning of gene sequences encoding proteins with HDAC activity has established the existence of a set of discrete HDAC enzyme isoforms. Grozinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast  
25 Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC-1, HDAC-2, and HDAC-3 proteins are members of the first class of HDACs, and discloses new proteins, named HDAC-4, HDAC-5, and HDAC-6, which are

members of the second class of HDACs. Kao *et al.*, *Gene & Development* **14**:55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al.* *J. Bio. Chem.* **275**:15254-13264 (2000) discloses the newest member of the first class of histone deacetylases, HDAC-8. It has been  
5 unclear what roles these individual HDAC enzymes play.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation for some time. Yoshida *et al.*, *J. Biol. Chem.* **265**:17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent inhibitor of mammalian HDAC. Yoshida *et al.*, *Cancer Res.* **47**:3688-3691 (1987)  
10 discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

Known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone  
15 deacetylase isoform, and more or less inhibit all members of both the histone deacetylase families equally. (Grozinger, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**:4868-4873 (1999)). For example, see Marks *et al.*, *J. National Cancer Inst.* **92**:1210-1216 (2000), which reviews histone deacetylase inhibitors and their role in studying differentiation and apoptosis.

20 Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to modulate the activity of specific histone deacetylase isoforms and to identify those isoforms involved in tumorigenesis and other proliferative diseases and disorders.

25

### **BRIEF SUMMARY OF THE INVENTION**

The invention provides methods and reagents for modulating the activity of histone deacetylase (HDAC) isoforms. For example, the invention provides methods and reagents for inhibiting HDAC isoforms, particularly HDAC-1 and HDAC-4, by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention provides for the specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further provides for the specific inhibition of particular HDAC isoforms involved in cell proliferation, and thus provides a treatment for cell proliferative diseases and disorders.

The inventors have made the surprising discovery that the specific inhibition of HDAC-4 dramatically induces apoptosis and growth arrest in cancerous cells. Accordingly, in a first aspect, the invention provides agents that inhibit the activity of the HDAC-4 isoform.

In certain preferred embodiments of the first aspect of the invention, the agent that inhibits the HDAC-4 isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding the HDAC-4 isoform. The nucleic acid molecule encoding the HDAC-4 isoform may be genomic DNA (e.g., a gene), cDNA, or RNA. In some embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC-4 isoform. In other embodiments, the oligonucleotide inhibits translation of the HDAC-4 isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule.

In a preferred embodiment thereof, the agent of the first aspect of the invention is an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4 or to a region of double-stranded DNA

that encodes a portion of HDAC-4. In one embodiment thereof, the antisense oligonucleotide is a chimeric oligonucleotide. In another embodiment thereof, the antisense oligonucleotide is a hybrid oligonucleotide. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence  
5 of from about 13 to about 35 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In still yet another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In another embodiment thereof, the antisense oligonucleotide has a nucleotide  
10 sequence of from about 20 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In still yet another embodiment thereof, the antisense oligonucleotide  
15 has a nucleotide sequence of from about 15 to about 26 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the antisense oligonucleotide  
20 is SEQ ID NO:11. In another embodiment thereof, the antisense oligonucleotide has one or more phosphorothioate internucleoside linkages. In another embodiment thereof, the antisense oligonucleotide further comprises a length of 20-26 nucleotides. In still another embodiment thereof, the antisense oligonucleotide is modified such that the terminal four  
25 nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2' -O- methyl groups attached to their sugar residues.

In certain preferred embodiments of the first aspect, the agent that inhibits the HDAC-4 isoform in a cell is a small molecule inhibitor that inhibits expression of a nucleic acid molecule encoding HDAC-4 isoform or activity of the HDAC-4 protein.

- 5           In a second aspect, the invention provides a method for inhibiting HDAC-4 activity in a cell, comprising contacting the cell with a specific inhibitor of HDAC-4, whereby HDAC-4 activity is inhibited. In an embodiment thereof, the invention provides method for inhibiting the HDAC-4 isoform in a cell, comprising contacting the cell with an antisense
- 10   oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4 or to a region of double-stranded DNA that encodes a portion of HDAC-4, whereby HDAC-4 activity is inhibited. In one embodiment thereof, the cell is contacted with an HDAC-4 antisense oligonucleotide that is a chimeric oligonucleotide. In another embodiment thereof, the cell is
- 15   contacted with an HDAC-4 antisense oligonucleotide that is a hybrid oligonucleotide. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In still yet another embodiment thereof, the antisense oligonucleotide has a nucleotide
- 20   sequence of from about 15 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In yet another embodiment thereof, the cell is contacted with an HDAC-4 antisense
- 25   oligonucleotide that has a nucleotide sequence length of from about 13 to about 35 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the cell is contacted with an HDAC-4

antisense oligonucleotide that has a nucleotide sequence length of from about 15 to about 26 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the cell is contacted with an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11. In another  
5 embodiment thereof, the inhibition of HDAC-4 activity leads to the inhibition of cell proliferation in the contacted cell. In another embodiment thereof, the inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell. In another embodiment thereof, the inhibition of HDAC-4 activity in the contacted cell further leads to growth  
10 arrest of the contacted cell. In another embodiment thereof, the inhibition of HDAC-4 activity in the contacted cell further leads to programmed cell death of the contacted cell. In another embodiment thereof, the inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the contacted cell. In certain embodiments thereof, the cell is a neoplastic cell  
15 which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method further comprises contacting the cell with an HDAC-4 small molecule inhibitor that interacts with and reduces the enzymatic activity of the HDAC-4 histone deacetylase isoform. In some embodiments thereof, the histone deacetylase  
20 small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal, comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically  
25 effective amount of a specific inhibitor of HDAC-4, whereby neoplastic cell proliferation is inhibited in the animal. In an embodiment thereof, the invention provides a method for inhibiting neoplastic cell growth in an



animal, comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the antisense oligonucleotide of the first aspect of the invention with a pharmaceutically acceptable carrier for a therapeutically effective period of time. In an

5 embodiment thereof, the animal is administered a chimeric HDAC-4 antisense oligonucleotide. In another embodiment thereof, the animal is administered a hybrid HDAC-4 antisense oligonucleotide. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides selected from the nucleotide sequence of SEQ ID NO:4.

10 In still yet another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:4. In

15 another embodiment thereof, the animal is administered an HDAC-4 antisense oligonucleotide having a nucleotide sequence of from about 13 to about 35 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the animal is administered an HDAC-4 antisense oligonucleotide having a nucleotide sequence of from

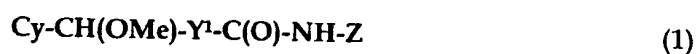
20 about 15 to about 26 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:11. In another embodiment thereof, the animal is administered an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11. In another embodiment thereof, the animal is a human. In another embodiment thereof, the method further comprises administering to an animal a

25 therapeutically effective amount of an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-1 or double-stranded DNA that encodes a portion of HDAC-1. In an embodiment

thereof, the animal is administered a chimeric HDAC-1 antisense oligonucleotide. In another embodiment thereof, the animal is administered a hybrid HDAC-1 antisense oligonucleotide. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides selected from the nucleotide sequence of SEQ ID NO:2. In still yet another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:2. In another embodiment thereof, the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides selected from the nucleotide sequence of SEQ ID NO:2. In another embodiment thereof, the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 13 to about 35 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:5. In another embodiment thereof, the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 15 to about 26 nucleotides and which comprises the nucleotide sequence of SEQ ID NO:5. In yet another embodiment thereof, the animal is administered an HDAC-1 antisense oligonucleotide that is SEQ ID NO:5.

In fourth aspect, the invention provides a method for inhibiting HDAC-4 activity in a cell, comprising contacting the cell with a small molecule inhibitor of HDAC-4, wherein HDAC-4 activity is inhibited.

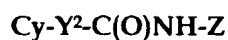
In one embodiment thereof, the cell is contacted with a small molecule inhibitor having the structure



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene

optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the anilinyll or  
5 pyridyl or thiadiazolyl may be optionally substituted.

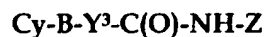
In another embodiment thereof, the invention provides a method wherein the cell is contacted with a small molecule inhibitor having the structure



10 (2)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group  
15 consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is anilinyll or pyridyl, or thiadiazolyl, any of which may be optionally substituted.

In another embodiment thereof, the invention provides a method wherein the cell is contacted with a small molecule inhibitor having the  
20 structure



(3)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; B is selected from the group consisting of -  
25 CH(OMe), ketone and methylene; Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms

of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the anilinyll or pyridyl or thiadiazolyl may be optionally substituted.

In another embodiment thereof, the invention provides a method wherein the cell is contacted with a small molecule inhibitor having the structure



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, wherein said alkylene may be optionally substituted; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when L<sup>1</sup> is -C(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>-, n being 1, 2, or 3, and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indolinyll.

In another embodiment thereof, the invention provides a method wherein the cell is contacted with a small molecule inhibitor having the structure



5 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be

optionally substituted, provided that Cy is not a

(spirocycloalkyl)heterocyclyl; L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub>

alkenylene, wherein the alkylene or alkenylene optionally may be

10 substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR', R' being alkyl, acyl, or

hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or

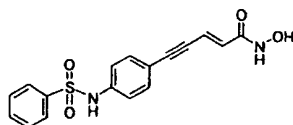
15 heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>2</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, which may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula -C(O)R wherein R comprises an α-amino  
20 acyl moiety; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when the carbon atom to which Cy is attached is oxo substituted, then Cy and Z are not both pyridyl.

In another embodiment thereof, the invention provides a method

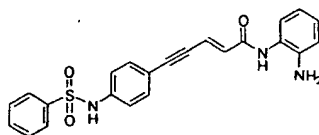
25 wherein the cell is contacted with a small molecule inhibitor has the structure



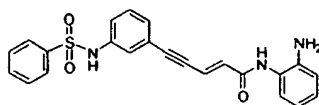
- wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>3</sup> is selected from the group consisting of (a) -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; and (b) C<sub>1</sub>-C<sub>6</sub> alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>3</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>3</sup> is C<sub>2</sub> alkenylene or C<sub>2</sub> alkynylene, wherein one or both carbon atoms of the alkenylene optionally may be substituted with alkyl, aryl, alkaryl, or aralkyl; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when Cy is unsubstituted phenyl, Ar is not phenyl wherein L<sup>3</sup> and Y<sup>3</sup> are oriented *ortho* or *meta* to each other.
- In another embodiment thereof, the invention provides a method wherein the cell is contacted with a small molecule inhibitor having the structure selected from the group consisting of



(7)



(8) and

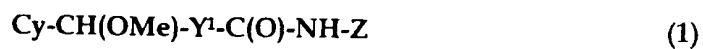


(9).

In another embodiment therein, the invention provides a method wherein the inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell. In another embodiment therein, the invention provides a method wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell. In another embodiment therein, the invention provides a method wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell. In another embodiment therein, the invention provides a method wherein inhibition of HDAC-4 activity in the contacted cell further leads to programmed cell death of the contacted cell. In another embodiment therein, the invention provides a method wherein inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the contacted cell. In another embodiment thereof, the contacted cell is a human cell.

In fifth aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal, comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of a small molecule inhibitor of HDAC-4, whereby neoplastic cell proliferation is inhibited. In one embodiment thereof, the animal is administered a small molecule inhibitor having the

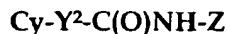
structure



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may  
5 be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the anilinyll or  
10 pyridyl or thiadiazolyl may be optionally substituted. In



another embodiment thereof, the invention provides a method wherein the animal is administered a small molecule inhibitor having the structure

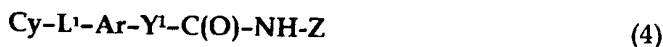


(2)

- 5 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene, wherein said alkylene be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z
- 10 is anilinyll or pyridyl or thiadiazolyl, any of which may be optionally substituted. In another embodiment thereof, the invention provides a method wherein the animal is administered a small molecule inhibitor having the structure



- 15 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; B is selected from the group consisting of -CH(OMe), ketone and methylene; Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected
- 20 from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the anilinyll or pyridyl or thiadiazolyl may be optionally substituted. In another embodiment thereof, the invention provides a
- 25 method wherein the animal is administered a small molecule inhibitor having the structure

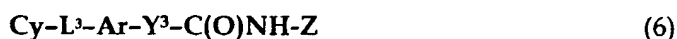


wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, wherein said alkylene may be optionally substituted; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when L<sup>1</sup> is -C(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>-, n being 1, 2, or 3, and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indolyl. In another embodiment thereof, the invention provides a method wherein the animal is administered a small molecule inhibitor having the structure



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR', R' being alkyl, acyl, or

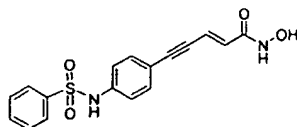
hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>2</sup> is a  
 5 chemical bond or a straight- or branched-chain saturated alkylene, which may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula -C(O)R wherein R comprises an α-amino acyl moiety; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation;  
 10 provided that when the carbon atom to which Cy is attached is oxo substituted, then Cy and Z are not both pyridyl. In another embodiment thereof, the invention provides a method wherein the animal is administered a small molecule inhibitor having the structure



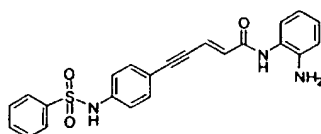
15 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>3</sup> is selected from the group consisting of (a) -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and  
 20 -NH-C(O)-NH-; and (b) C<sub>1</sub>-C<sub>6</sub> alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>3</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted  
 25 and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>3</sup> is C<sub>2</sub> alkenylene or C<sub>2</sub> alkynylene, wherein

one or both carbon atoms of the alkenylene optionally may be substituted with alkyl, aryl, alkaryl, or aralkyl; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when Cy is unsubstituted  
5 phenyl, Ar is not phenyl wherein L<sup>3</sup> and Y<sup>3</sup> are oriented *ortho* or *meta* to each other. In another embodiment thereof, the invention provides a method wherein the animal is

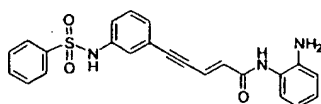
administered a small molecule inhibitor having the structure selected from the group consisting of



(7)



(8) and



(9).

5

In another embodiment thereof, the invention provides a method wherein the animal administered a small molecule inhibitor is a human.

In a sixth aspect, the invention provides a method for inhibiting the induction of cell proliferation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of HDAC-4 and/or contacting a cell with a small molecule inhibitor of HDAC-4. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis.

In a seventh aspect, the invention provides a method for identifying a small molecule histone deacetylase inhibitor that inhibits the HDAC-4 isoform, the isoform being required for the induction of cell proliferation. The method comprises contacting the HDAC-4 isoform with a candidate small molecule inhibitor and measuring the enzymatic activity of the contacted histone deacetylase isoform, wherein a reduction in the enzymatic activity of

15

the contacted HDAC-4 isoform identifies the candidate small molecule inhibitor as a small molecule histone deacetylase inhibitor of the HDAC-4 isoform.

5 In an eighth aspect, the invention provides a method for identifying a small molecule histone deacetylase inhibitor that inhibits HDAC-4 isoform, which is involved in the induction of cell proliferation. The method comprises contacting a cell with a candidate small molecule inhibitor and measuring the enzymatic activity of the contacted histone deacetylase isoform, wherein a reduction in the enzymatic activity of the HDAC-4 isoform  
10 identifies the candidate small molecule inhibitor as a small molecule histone deacetylase inhibitor of HDAC-4.

In a ninth aspect, the invention provides a small molecule histone deacetylase inhibitor identified by the method of the seventh or the eighth aspect of the invention. Preferably, the histone deacetylase small molecule  
15 inhibitor is substantially pure.

In a tenth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising, contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits expression of HDAC-4 isoform, a small molecule histone deacetylase  
20 inhibitor that inhibits expression or activity of HDAC-4 isoform, an antisense oligonucleotide that inhibits expression of the HDAC-1 isoform, a small molecule histone deacetylase inhibitor that inhibits the expression or the activity of the HDAC-1 isoform, an antisense oligonucleotide that inhibits expression of a DNA methyltransferase, and a small molecule DNA  
25 methyltransferase inhibitor. In certain embodiments, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of

the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional embodiments, the reagents selected from the group are operably associated.

- In an eleventh aspect, the invention provides a method of inhibiting
- 5 neoplastic cell growth, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits expression of HDAC-4 isoform, a small molecule histone deacetylase inhibitor that inhibits the expression or the activity of HDAC-4 isoform, an
- 10 antisense oligonucleotide that inhibits expression of the HDAC-1 isoform, a small molecule histone deacetylase inhibitor that inhibits expression or activity of the HDAC-1 isoform, an antisense oligonucleotide that inhibits expression of a DNA methyltransferase, and a small molecule DNA
- 15 methyltransferase inhibitor. In some embodiments, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred
- embodiments, the cell is a neoplastic cell. In yet additional preferred
- embodiments, the reagents selected from the group are operably associated.

### BRIEF DESCRIPTION OF THE DRAWINGS

- 5 Fig. 1 AS1 and AS2 can inhibit HDAC4 expression at RNA level in a dose-dependent manner. Human cancer A549 cells were treated with escalating doses of AS1, AS2 or MM2 oligos for 24 hours. Total RNAs were harvested for Northern analysis.
- 10 Fig. 2 AS1 and AS2 can inhibit HDAC4 expression at protein level. Human cancer A549 cells were treated with AS1, AS2 or MM2 oligos for 48 hours. Whole cell lysates were analyzed by Western blotting using antibodies specific against human HDAC4.
- 15 Fig. 3 Growth curve of human cancer cells A549 treated with HDAC4 AS1 or AS2. Cells were plated at  $2.5 \times 10^5$ /10 cm dish at 0 hour time point. Cells were treated with 50 nM oligos at 24 and 48 hours. Cells were counted at 24, 48 and 72 hours by trypan blue exclusion.
- 20 Fig. 4 Growth curve of human cancer cells Du145 treated with HDAC4 AS1 or AS2. Cells were plated at  $2.5 \times 10^5$ /10 cm dish at day 0. Cells were treated with 50 nM oligos at day 1, day 2 and day 3. Cells were counted at day 1, day 2, day 3 and day 4 by trypan blue exclusion.
- 25 Fig. 5 Graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.
- 30 Figure 6 is a graphic representation demonstrating the cell cycle blocking effect of HDAC-4 antisense oligos on human A549 cancer cells.
- Figure 7 is a representation of an RNase protection assay demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype mRNA expression in human A549 cells.



Figure 8 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 antisense oligos induces the expression of the p21 protein.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5           The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

10           The invention provides methods and reagents for modulating histone deacetylase (HDAC) isoforms, particularly HDAC-1 and HDAC-4, by inhibiting expression at the nucleic acid level or by inhibiting enzymatic activity at the protein level. The invention provides for the specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis, and thus  
15 provides a treatment for cancer. The invention further provides for the specific inhibition of specific HDAC isoforms involved in cell proliferation and thus provides a treatment for cell proliferative disorders.

          The inventors have made the surprising discovery that the specific inhibition of HDAC-4 dramatically induces apoptosis and growth arrest in  
20 cancerous cells. This discovery has been exploited to develop the present invention which, in a first aspect, provides agents that inhibit the HDAC-4 isoform.

          In certain preferred embodiments of the first aspect of the invention, the agent that inhibits the HDAC-4 isoform is an oligonucleotide that inhibits  
25 expression of a nucleic acid molecule encoding HDAC-4 isoform. The HDAC-4 nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA.

In some embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC-4 isoform. In other embodiments, the oligonucleotide inhibits translation of the HDAC-4 isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule.

- 5 Preferred antisense oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

In certain preferred embodiments, the agent that inhibits the HDAC-4 isoform is a small molecule inhibitor that inhibits expression of a nucleic acid molecule encoding HDAC-4 isoform or activity of the HDAC-4 protein.

- 10 The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule
- 15 encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least
- 20 about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In a particularly preferred embodiment, the small molecule inhibitor of HDAC is an inhibitor of HDAC-1 and/or HDAC-4. Most preferred are small molecule inhibitors of HDAC-4.

- 25 Preferably, such inhibition is specific, *i.e.*, the histone deacetylase inhibitor reduces the ability of a histone deacetylase to remove an acetyl group from a histone at a concentration that is lower than the concentration of

the inhibitor that is required to produce another, unrelated biological effect. Preferably, the concentration of the inhibitor required for histone deacetylase inhibitory activity is at least 2-fold lower, more preferably at least 5-fold lower, even more preferably at least 10-fold lower, and most preferably at least 20-fold lower than the concentration required to produce an unrelated biological effect.

Preferred agents that inhibit HDAC-4 inhibit growth of human cancer cells, independent of their p53 status. These agents induce apoptosis in cancer cells and cause growth arrest. They also can induce transcription of p21<sup>WAF1</sup> (a tumor suppressor gene), *Bax*, an extremely important gene involved in apoptosis regulation and *GADD45*, a stress-induced gene and important regulator of cell growth. These agents may exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention.

The antisense oligonucleotides according to the invention are complementary to a region of RNA or to a region of double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform). For purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleosides, ribonucleosides, or any combination thereof. Preferably, such oligonucleotides have from about 6 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate,

phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleotide linkages. These internucleoside linkages preferably are  
5 phosphotriester, phosphorothioate, or phosphoramidate linkages, or combinations thereof.

Preferably, the oligonucleotides may also contain 2'-O-substituted ribonucleotides. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower  
10 alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl, or allyl group may be unsubstituted or may be substituted, *e.g.*, with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy  
15 group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group. The term "alkyl" as employed herein refers to straight and branched chain aliphatic groups having from 1 to 12 carbon atoms, preferably 1-8 carbon atoms, and more preferably 1-6 carbon atoms, which may be optionally substituted with one, two or three substituents. Unless otherwise  
20 apparent from context, the term "alkyl" is meant to include saturated, unsaturated, and partially unsaturated aliphatic groups. When unsaturated groups are particularly intended, the terms "alkenyl" or "alkynyl" will be used. When only saturated groups are intended, the term "saturated alkyl" will be used. Preferred saturated alkyl groups include, without limitation,  
25 methyl, ethyl, propyl, isopropyl, butyl, isobutyl, *sec*-butyl, *tert*-butyl, pentyl, and hexyl.

The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents including, without limitation, lipophilic groups, intercalating agents, diamines, and adamantane. The term oligonucleotide also encompasses such  
5 polymers as PNA and LNA.

For purposes of the invention, the term "complementary" means having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof, under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between  
10 complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

15 Particularly preferred antisense oligonucleotides utilized in this aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One  
20 preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising internucleoside linkages, phosphorothioate, phosphorodithioate, internucleoside linkages and phosphodiester, preferably comprising from about 2 to about 12 nucleotides. Some useful oligonucleotides of the invention have an alkylphosphonate-linked region  
25 and an alkylphosphonothioate region (see *e.g.*, Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain

at least three consecutive internucleoside linkages that are phosphodiester and phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred  
5 embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-O-substituted ribonucleotide region, preferably comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide contains at least three consecutive deoxyribonucleosides and contains ribonucleosides, 2'-O-substituted  
10 ribonucleosides, or combinations thereof (see *e.g.*, Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to modulate expression of the target  
15 sequence, *e.g.*, the HDAC-4 or the HDAC-1 isoform. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding the HDAC-4 or the HDAC-1 isoform, quantitating the amount of the HDAC-4 or the HDAC-1 isoform protein, quantitating the the HDAC-4 or the HDAC-1  
20 enzymatic activity, or quantitating the ability of the the HDAC-4 or the HDAC-1 isoform, for example, to inhibit cell growth in a an *in vitro* or *in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit expression" and similar terms used herein are intended to encompass any one or more of these parameters.

25 Antisense oligonucleotides according to the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry, phosphoramidite

chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase

5 oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*, Pon, R. T., *Meth. Molec. Biol.* 20:465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used

10 to inhibit the activity of specific histone deacetylase isoforms in an experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform

15 expression according to the invention and observing any phenotypic effects. In this use, the antisense oligonucleotides used according to the invention are preferable to traditional "gene knockout" approaches because they are easier to use, and because they can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

20 Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the the HDAC-4 or the HDAC-1 isoform, and/or the translation of a nucleic acid molecule encoding the the HDAC-4 or the HDAC-1, and/or lead to the degradation of such nucleic acid molecules. HDAC-4- or HDAC-1-encoding nucleic acid

25 molecules may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5' and 3' regions, intron-exon boundaries, as well as coding sequences from the HDAC-4 or the

HDAC-1 isoform genes. For human sequences, see *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA* 93(23):2845-12850, 1996; Furukawa *et al.*, *Cytogenet. Cell Genet.* 73(1-2):130-133, 1996; Yang *et al.*, *J. Biol. Chem.* 272(44):28001-28007, 1997; Betz *et al.*, *Genomics* 52(2):245-246, 1998; Taunton *et al.*, *Science* 272(5260):408-411, 5 1996; and Dangond *et al.*, *Biochem. Biophys. Res. Commun.* 242(3):648-652, 1998).

Antisense oligonucleotides for human HDAC isotype polynucleotides may be designed from known HDAC isotype sequence data. For example, the following amino acid sequences are available from GenBank for HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, and HDAC-8: 10 AAC50475, AAC50814, AAC98927, BAA22957, AB011172, AAD29048, AAF63491, and AAF73076, respectively, and the following nucleotide sequences are available from GenBank for HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, and HDAC-8: U50079, U31814, AF039703, AB006626, AF039691, AJ011972, AF239243, and AF230097, 15 respectively.

Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to a region of RNA or to a region of double-stranded DNA encoding the HDAC-4 or the HDAC-1 isoform, (see *e.g.*, GenBank Accession No. U50079 for human HDAC-1 (Fig. 20 1B), and GenBank Accession No. AB006626 for human HDAC-4 (Fig. 2B)).

The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Nos. X98207 (murine HDAC-1) and AF006602 (murine HDAC-4)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to 25 a region of RNA or to a region of double-stranded DNA that encode the HDAC-4 or the HDAC-1 isoform from non-human animals. Antisense



oligonucleotides according to these embodiments are useful as tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences  
5 shown in Table I below.

These oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below in Table I. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the  
10 terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2' -O- methyl groups attached to their sugar residues.

Table 1: HDAC isotype-specific antisense and mismatch oligos

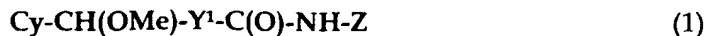
Oligo	Target	Accession Number	Nucleotide Position	Sequence	position within Gene
HDAC1 AS1	Human HDAC1	U50079	1585-1604	5'-GAAACGTGAGGGACTCAGCA-3'	3'-UTR
HDAC1 AS2	Human HDAC1	U50079	1565-1584	5'-GGAAGCCAGAGCTGGAGAGG-3'	3'-UTR
HDAC1 MM	Human HDAC1	U50079	1585-1604	5'-GTTAGGTGAGGCACTGAGGA-3'	3'-UTR
HDAC2 AS	Human HDAC2	U31814	1643-1622	5'-GCTGAGCTGTTCTGATTTGG-3'	3'-UTR
HDAC2 MM	Human HDAC2	U31814	1643-1622	5'-CGTGAGCACTTCTCATTTC-3'	3'-UTR
HDAC3 AS	Human HDAC3	AF039703	1276-1295	5'-CGCTTTCCTTGTCATTGACA-3'	3'-UTR
HDAC3 MM	Human HDAC3	AF039703	1276-1295	5'-GCCTTTCCTACTCATTGTGT-3'	3'-UTR
HDAC4 AS1	Human HDAC4	AB006626	514-33	5'-GCTGCCTGCCGTGCCACCC-3'	5'-UTR
HDAC4 MM1	Human HDAC4	AB006626	514-33	5'-CGTGCCTGCGCTGCCACGG-3'	5'-UTR
HDAC4	Human	AB006626	7710-29	5'-TACAGTCCATGCAACCTCCA-	3'-UTR

AS2 HDAC4 MM2	Human HDAC4	AB006626	7710-29	5'-ATCAGTCCAACCAACCTCGT-3'	3'-UTR
HDAC5 AS1	Human HDAC5	BE794912	1-20	5'-GCAGCGGCGGCAGCACCTCC-3'	5'-UTR
HDAC5 AS2	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGCTTGG-3'	3'-UTR
HDAC6 AS	Human HDAC6	AJ011972	3791-3810	5'-CAGGCTGGAATGAGCTACAG-3'	3'-UTR
HDAC6 MM	Human HDAC6	AJ011972	3791-3810	5'-GACGCTGCAATCAGGTAGAC-3'	3'-UTR
HDAC7 AS1	Human HDAC7	AF239243	65-84	5'-CAGGCTCACTTGACAATGGC-3'	5'-UTR
HDAC7 AS2	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCCACA-3'	3'-UTR
HDAC8 AS1	Human HDAC8	AF230097	51-70	5'-CTCCGGCTCCTCCATCTTCC-3'	5'-UTR
HDAC8 AS2	Human HDAC8	AF230097	1328-1347	5'-AGCCAGCTGCCACTTGATGC-3'	3'-UTR

The antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents (see preparation of pharmaceutically acceptable formulations in, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the proviso that such carriers or diluents not affect their ability to modulate HDAC activity.

In certain preferred embodiments, the agent that inhibits the HDAC-4 and/or HDAC-1 isoform is a small molecule. In certain preferred embodiments, the small molecule inhibits the enzymatic activity of the HDAC-4 or HDAC-1 isoform.

Certain preferred small molecule inhibitors of the HDAC-4 and/or HDAC-1 isoform include compounds having the formula (1):



wherein:

- 5            Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may optionally be substituted;

             Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene which optionally may be substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety such as O, NR<sup>1</sup> (R<sup>1</sup> being alkyl, acyl or hydrogen) S,  
10        S(O), or S(O)<sub>2</sub>; and

             Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted.

- An "alkylene" group is an alkyl group, as defined hereinabove, that is  
15        positioned between and serves to connect two other chemical groups. Preferred alkylene groups include, without limitation, methylene, ethylene, propylene, and butylene.

- The term "cycloalkyl" as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons,  
20        preferably 3 to 8 carbons, and more preferably 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Preferred cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

- 25            An "aryl" group is a C<sub>6</sub>-C<sub>14</sub> aromatic moiety comprising one to three aromatic rings, which may be optionally substituted. Preferably, the aryl

group is a C<sub>6</sub>-C<sub>10</sub> aryl group. Preferred aryl groups include, without limitation, phenyl, naphthyl, anthracenyl, and fluorenyl. An "aralkyl" or "arylalkyl" group comprises an aryl group covalently linked to an alkyl group, either of which may independently be optionally substituted or  
5 unsubstituted. Preferably, the aralkyl group is (C<sub>1</sub>-C<sub>6</sub>)alk(C<sub>6</sub>-C<sub>10</sub>)aryl, including, without limitation, benzyl, phenethyl, and naphthylmethyl. An "alkaryl" or "alkylaryl" group is an aryl group having one or more alkyl substituents. Examples of alkaryl groups include, without limitation, tolyl, xylyl, mesityl, ethylphenyl, *tert*-butylphenyl, and methylnaphthyl.

10 An "arylene" group is an aryl group, as defined hereinabove, that is positioned between and serves to connect two other chemical groups. Preferred arylene groups include, without limitation, phenylene and naphthylene. The term "arylene" is also meant to include heteroaryl bridging groups, including, but not limited to, benzothienyl, benzofuryl, quinolyl,  
15 isoquinolyl, and indolyl.

A "heterocyclyl" or "heterocyclic" group is a ring structure having from about 3 to about 8 atoms, wherein one or more atoms are selected from the group consisting of N, O, and S. The heterocyclic group may be optionally substituted on carbon at one or more positions. The heterocyclic group may  
20 also independently be substituted on nitrogen with alkyl, aryl, aralkyl, alkylcarbonyl, alkylsulfonyl, arylcarbonyl, arylsulfonyl, alkoxycarbonyl, aralkoxycarbonyl, or on sulfur with oxo or lower alkyl. Preferred heterocyclic groups include, without limitation, epoxy, aziridinyl, tetrahydrofuranyl, pyrrolidinyl, piperidinyl, piperazinyl, thiazolidinyl, oxazolidinyl,  
25 oxazolidinonyl, and morpholino. In certain preferred embodiments, the heterocyclic group is fused to an aryl or heteroaryl group. Examples of such

fused heterocycles include, without limitation, tetrahydroquinoline and dihydrobenzofuran.

As used herein, the term "heteroaryl" refers to groups having 5 to 14 ring atoms, preferably 5, 6, 9, or 10 ring atoms; having 6, 10, or 14  $\pi$  electrons shared in a cyclic array; and having, in addition to carbon atoms, between one and about three heteroatoms selected from the group consisting of N, O, and S. Preferred heteroaryl groups include, without limitation, thienyl, benzothienyl, furyl, benzofuryl, dibenzofuryl, pyrrolyl, imidazolyl, pyrazolyl, pyridyl, pyrazinyl, pyrimidinyl, indolyl, quinolyl, isoquinolyl, quinoxaliny, tetrazolyl, oxazolyl, thiazolyl, and isoxazolyl.

As employed herein, a "substituted" alkyl, cycloalkyl, aryl, heteroaryl, or heterocyclic group is one having between one and about four, preferably between one and about three, more preferably one or two, non-hydrogen substituents. Suitable substituents include, without limitation, halo, hydroxy, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups.

The term "halogen" or "halo" as employed herein refers to chlorine, bromine, fluorine, or iodine.

As herein employed, the term "acyl" refers to an alkylcarbonyl or arylcarbonyl substituent.

The term "acylamino" refers to an amide group attached at the nitrogen atom. The term "carbamoyl" refers to an amide group attached at the carbonyl carbon atom. The nitrogen atom of an acylamino or carbamoyl substituent may be additionally substituted. The term "sulfonamido" refers to

a sulfonamide substituent attached by either the sulfur or the nitrogen atom. The term "amino" is meant to include NH<sub>2</sub>, alkylamino, arylamino, and cyclic amino groups.

The term "ureido" as employed herein refers to a substituted or  
5 unsubstituted urea moiety.

In another embodiment, the small molecule inhibitors of the HDAC-4 and/or HDAC-1 isoform are represented by formula (2):



(2)

10 wherein:

Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may optionally be substituted;

Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene which may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a  
15 heteroatom moiety such as O, NR<sup>1</sup> (R<sup>1</sup> being alkyl, acyl or hydrogen), S, S(O), or S(O)<sub>2</sub>; and

Z is anilinyll or pyridyl or thiadiazolyl, any of which may optionally be optionally substituted. In another embodiment, preferred small molecule inhibitors of the HDAC-4 and/or HDAC-1 isoform include compounds  
20 having the formula (3):



wherein:

Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may optionally be substituted;

25 B is -CH(OMe), ketone, or methylene;

Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene which may be optionally substituted, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety such as O, NR<sup>1</sup> (R<sup>1</sup> being alkyl, acyl or hydrogen), S, S(O), or S(O)<sub>2</sub>; and

5           Z is aniliny, pyridyl, thiadiazolyl or -O-M (M being H or a pharmaceutically acceptable cation), wherein the aniliny or pyridyl or thiadiazolyl optionally may be substituted.

In another embodiment, the inhibitors of the HDAC-4 and/or HDAC-1 isoform are represented by formula (4):

$$10 \quad \text{Cy-L}^1\text{-Ar-Y}^1\text{-C(O)-NH-Z} \quad (4)$$

wherein:

Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which optionally may be substituted;

L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is -C(O)NH-, -

15  $\text{S(O)}_2\text{NH-}$ ,  $-\text{NHC(O)-}$ ,  $-\text{NHS(O)}_2-$ , or  $-\text{NH-C(O)-NH-}$ ;

Ar is arylene which may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which optionally may be substituted;

20 Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated  
alkylene, which optionally may be substituted; and

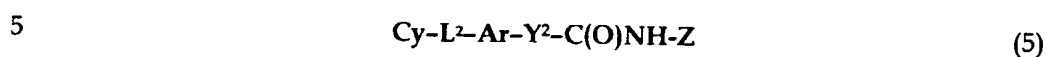
Z is aniliny, pyridyl, thiadiazolyl, or -O-M (M being H or a pharmaceutically acceptable cation);

provided that when L<sup>1</sup> is -C(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>- (n being 1, 2, or 3),

25 and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or

hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indolyl.

In another embodiment, the inhibitors of the HDAC-4 and/or HDAC-1 isoform are represented by formula (5):



wherein:

Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which optionally may be substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl;

10 L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety such as O, NR' (R' being alkyl, acyl, or hydrogen), S, S(O), or S(O)<sub>2</sub>;

15 Ar is arylene which optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which optionally may be substituted; and

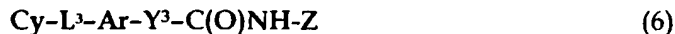
20 Y<sup>2</sup> is a chemical bond or a straight- or branched-chain saturated alkylene which may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula -C(O)R, wherein R comprises an α-amino acyl moiety; and

Z is aniliny, pyridyl, thiadiazolyl, or -O-M (M being H or a pharmaceutically acceptable cation);

25 provided that when the carbon atom to which Cy is attached is oxo-substituted, then Cy and Z are not both pyridyl.



In another embodiment, the inhibitors of the HDAC-4 and/or HDAC-1 isoform are represented by formula (6):



wherein:

- 5            Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which optionally may be substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl;

L<sup>3</sup> is:

- (a)    -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is -  
 10       C(O)NH-, S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, or -NH-C(O)-NH-; or  
 (b)    C<sub>1</sub>-C<sub>6</sub> alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>3</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by O; NR', R' being alkyl, acyl, or hydrogen; S; S(O);  
 15       or S(O)<sub>2</sub>;

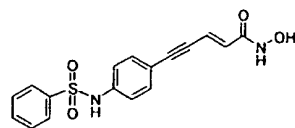
Ar is arylene which optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which optionally may be substituted; and

- 20           Y<sup>3</sup> is C<sub>2</sub> alkenylene or C<sub>2</sub> alkynylene, wherein one or both carbon atoms of the alkenylene optionally may be substituted with alkyl, aryl, alkaryl, or aralkyl; and

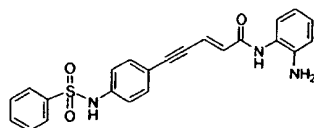
Z is anilinyl, pyridyl, thiadiazolyl, or -O-M (M being H or a pharmaceutically acceptable cation);

- 25           provided that when Cy is unsubstituted phenyl, Ar is not phenyl wherein L<sup>3</sup> and Y<sup>3</sup> are oriented *ortho* or *meta* to each other.

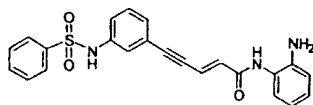
In another embodiment, the small molecule inhibitors of the HDAC-4 and/or HDAC-1 isoform have the structure selected from the group consisting of



(7)



(8) and



(9).

Non-limiting examples of small molecule inhibitors for use in the methods of the invention are presented in Table 2.

Table 2: Properties of Selected MG  
Anilides in vitro and in vivo (shown in uM)

MG #	Structure	Enzyme IC50 (uM)					H 4-Ac	MT T	cell cycle arrest EC	p21 induction	% inh. of tumor formation in vivo		
		HDA C1	HDA C2	HDA C3	HDA C4	HDA C6					colon	lung	prostate
24 29			25	21	23	>50	1	1	2	3			
36 50			4	>20	23	>50	10	5	9	10	53(40, ip)		
37 63			22	45	28	>50	5	4	2	2	55(40, ip)		
38 69			8	18	13	>50	5	5	3	5			

note: for in vivo antitumor studies, numbers outside brackets indicate % of inhibition of tumor formation in vivo;  
numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day);  
oral (PO) or intraperitoneal (IP) administration is indicated in brackets.

Small molecule inhibitors of the invention of the formulae Cy-

- 5 CH(OMe)-Y<sup>1</sup>-C(O)-NH-Z, Cy-Y<sup>2</sup>-C(O)NH-Z and Cy-B-Y<sup>3</sup>-C(O)-NH-Z, which may be conveniently prepared according to the following schemes 1-3 or using other art-recognized methods.

#### Scheme 1

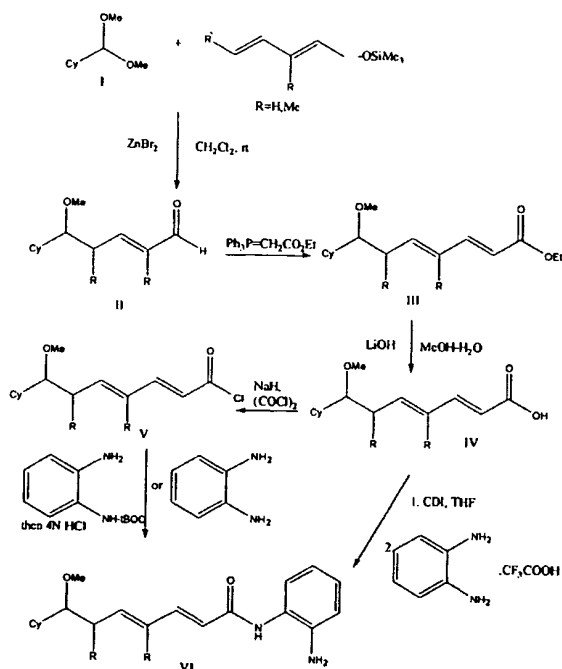
- A dialkyl acetal I is treated with 1-trimethylsilyloxy-1,3-butadiene or  
10 with 1-trimethylsilyloxy-2,4-dimethyl-1,3-butadiene in the presence of zinc

bromide to yield the aldehyde II. Wittig reaction of II with a carboalkoxy phosphorous ylide such as ethyl (triphenylphosphoranylidene)acetate yields the diene ester III. Hydrolysis of the ester function in III can be effected by treatment with a hydroxide base, such as lithium hydroxide, to yield the

5 corresponding acid IV.

The acid IV is converted to the corresponding acid chloride V according to standard methods, e.g., by treatment with sodium hydride and oxalyl chloride. Treatment of V with 1,2-phenylenediamine and a tertiary base such as *N*-methylmorpholine, preferably in dichloromethane at reduced

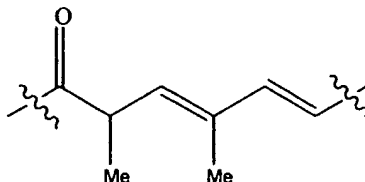
10 temperature, then yields the anilinyamide VI. Alternatively, the acid chloride



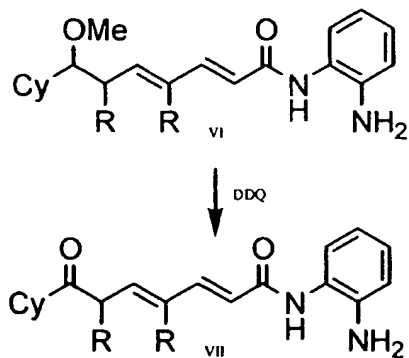
V may be treated with a mono-protected 1,2-phenylenediamine, such as 2-(*t*-BOC-amino)aniline, followed by deprotection, to yield VI.

In an alternative procedure, the acid IV may be activated by treatment with carbonyldiimidazole (CDI), followed by treatment with 1,2-phenylenediamine and trifluoroacetic acid, to yield the aniliny amide VI.

5 Compounds of formula  $\text{Cy-y}^2\text{-C(O)-NH}_2$  (VII), wherein  $\text{Y}^2$  is:

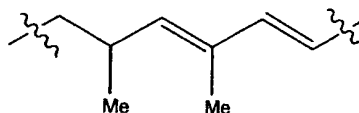


may be prepared from the corresponding methoxy-substituted compounds (VI) by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), as illustrated in Scheme 2



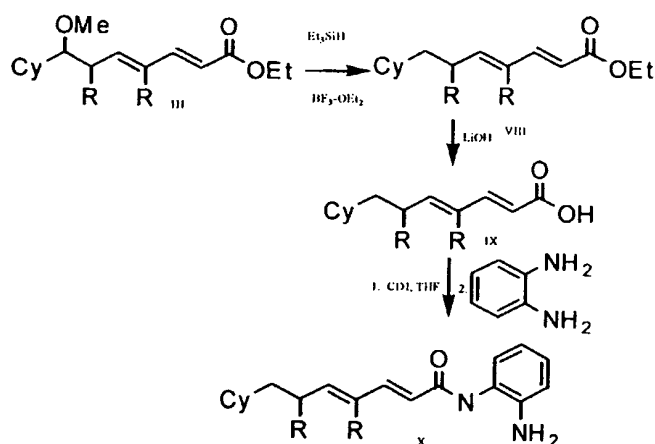
10

Compounds of formula  $\text{Cy-y}^2\text{-C(O)-NH}_2$ , wherein  $\text{y}^2$  has the structure



may be prepared as shown in Scheme 3. The methoxy substituted  
 5 diene ester **III**, prepared as described in Scheme 1, is treated with  
 triethylsilane and boron trifluoride etherate to yield the deoxygenated  
 compound **VIII**. Conversion of **VIII** to the anilinyllamide **X** is accomplished  
 by procedures analogous to those described in Scheme 1.

### Scheme 3

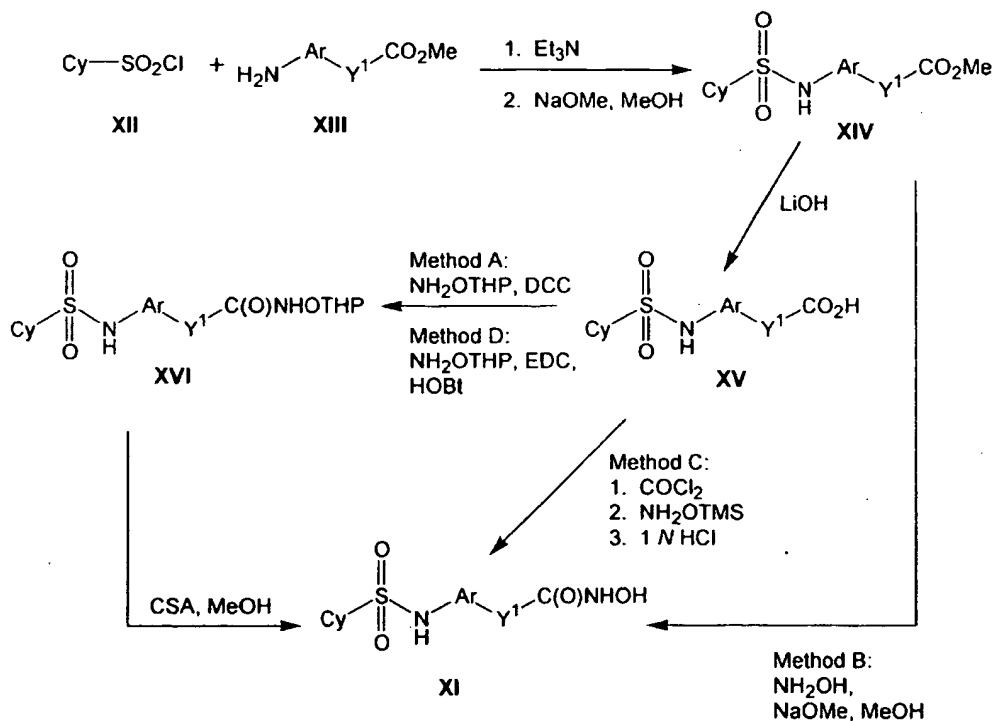


10

Compounds of formula  $\text{Cy-L}^1\text{-Ar-Y}^1\text{-C(O)-NH-O-M}$ , wherein  $\text{L}^1$  is  
 $-\text{S(O)}_2\text{NH}-$ , may be prepared according to the synthetic routes depicted in  
 Schemes 4-6. In certain preferred embodiments, compounds **XI** are preferably

- prepared according to the general synthetic route depicted in Scheme 4. A sulfonyl chloride (XII) is treated with an amine (XIII) in a solvent such as methylene chloride in the presence of an organic base such as triethylamine. Treatment of the crude product with a base such as sodium methoxide in an alcoholic solvent such as methanol effects cleavage of any dialkylated material and affords the sulfonamide (XIV). Hydrolysis of the ester function in XIV can be effected by treatment with a hydroxide base, such as lithium hydroxide, in a solvent mixture such as tetrahydrofuran and methanol to yield the corresponding acid (XV).

Scheme 4



In some embodiments, conversion of the acid XV to the hydroxamic acid XI is accomplished by coupling XV with a protected hydroxylamine, such as tetrahydropyranyloxyamine (NH<sub>2</sub>OTHP), to yield the protected hydroxamate XVI, followed by acidic hydrolysis of XVI to provide the hydroxamic acid XI. The coupling reaction is preferably accomplished with the coupling reagent dicyclohexylcarbodiimide (DCC) in a solvent such as methylene chloride (Method A), or with the coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in presence of *N*-hydroxybenzotriazole in an aprotic solvent such as dimethylformamide (Method D).

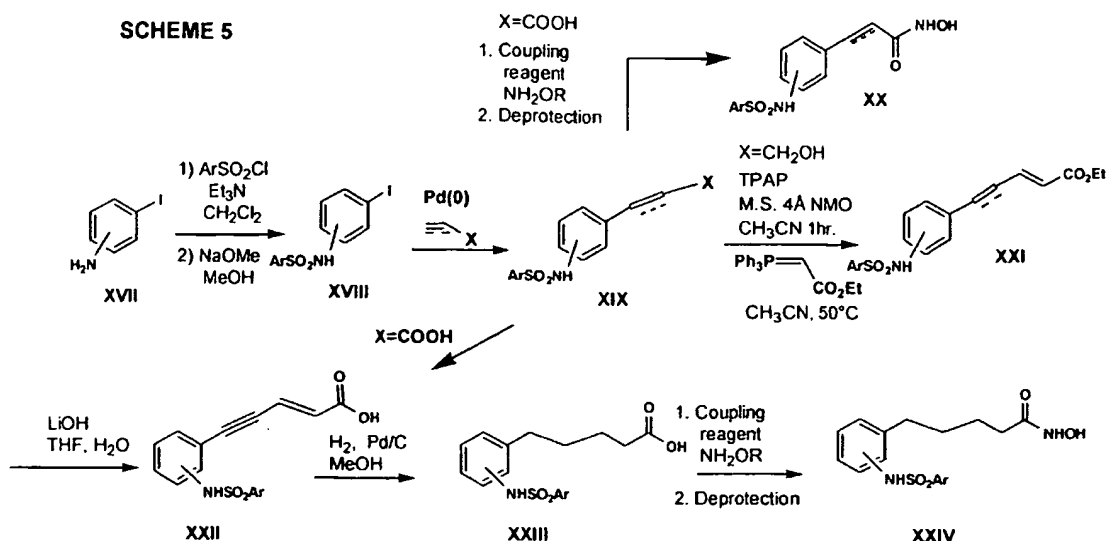
Other coupling reagents are known in the art and may also be used in this reaction. Hydrolysis of XVI is preferably effected by treatment with an organic acid such as camphorsulfonic acid in a protic solvent such as methanol.

Alternatively, in some other embodiments, acid XV is converted to the corresponding acid chloride, preferably by treatment with oxalyl chloride, followed by the addition of a protected hydroxylamine such as *O*-trimethylsilyloxyamine in a solvent such as methylene chloride, which then provides the hydroxamic acid XI upon workup (Method C).

In still other embodiments, the ester XIV is treated with hydroxylamine in a solvent such as methanol in the presence of a base such as sodium methoxide to furnish the hydroxamic acid XI directly (Method B).



SCHEME 5



Compounds of formula XX and XXIV preferably are prepared according to the general procedure outlined in Scheme 5 above.

5 An aminoaryl halide (XVII) is treated with a sulfonyl chloride in presence of a base such as triethylamine, followed by treatment with an alkoxide base, to furnish the sulfonamide XVIII. One of skill in the art will recognize that reverse sulfonamide analogs can be readily prepared by an analogous procedure, treating a haloarenesulfonyl halide with an arylamine.

10 Compound XVIII is coupled with a terminal acetylene or olefinic compound in the presence of a palladium catalyst such as tetrakis(triphenylphosphine)palladium(0) in a solvent such as pyrrolidine to yield XIX.

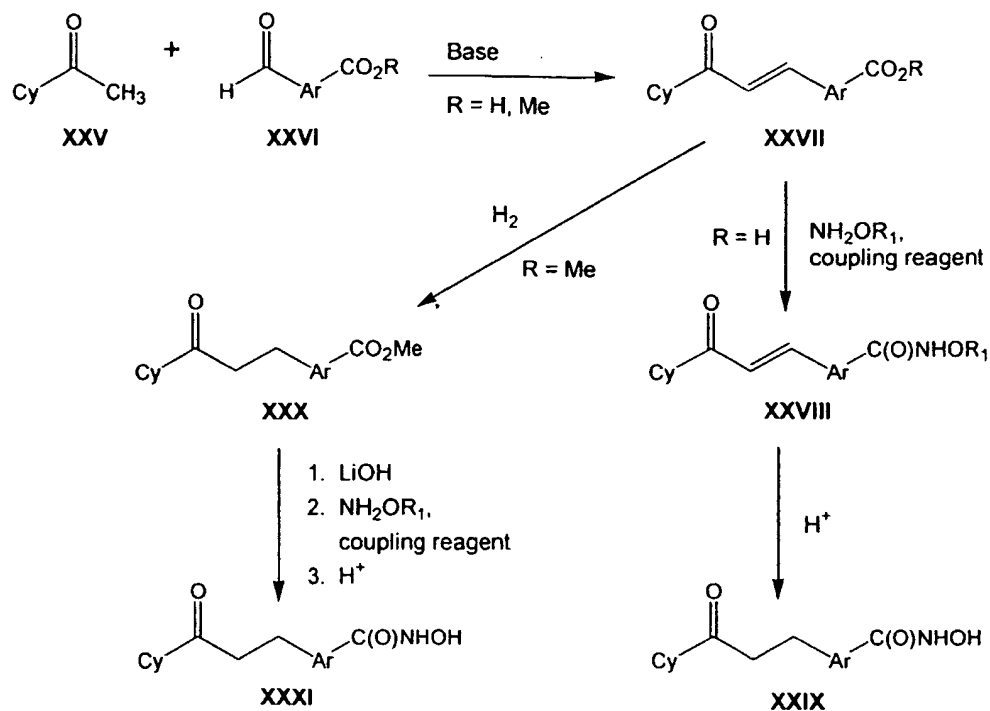
Oxidation of the compound of formula XIX ( $\text{X} = \text{CH}_2\text{OH}$ ), followed by  
 15 homologation of the resulting aldehyde (using a Wittig type reagent such as carbethoxymethylenetriphenylphosphorane in a solvent such as acetonitrile), yields the compound of formula XXI. Basic hydrolysis of XXI, such as by treatment with lithium hydroxide in a mixture of THF and water, provides

the acid XXII. Hydrogenation of XXII may preferably be performed over a palladium catalyst such as Pd/C in a protic solvent such as methanol to yield the saturated acid XXIII. Coupling of the acid XXIII with an *O*-protected hydroxylamine such as *O*-tetrahydropyranylhdroxylamine is effected by  
5 treatment with a coupling reagent such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in the presence of *N*-hydroxybenzotriazole (HOBT), or *N,N*-dicyclohexylcarbodiimide (DCC), in a solvent such as DMF, followed by deprotection to furnish the compound of general formula XXIV.

The acid XIX, wherein X=COOH, may be coupled directly with an  
10 *O*-protected hydroxylamine such as *O*-tetrahydropyranylhdroxylamine, followed by deprotection of the hydroxy protecting group to furnish the hydroxamic acid XX.

Compounds of formula Cy-L<sup>1</sup>-Ar-Y<sup>1</sup>-C(O)-NH-O-M, wherein L<sup>1</sup> is  
15 -C(O)NH-, preferably may be prepared according to the synthetic routes analogous to those depicted in Schemes 4-5, substituting acid chloride starting materials for the sulfonyl chloride starting materials in those schemes.

Scheme 6



5 Compounds of the formula  $Cy-L^2-Ar-Y^2-C(O)-NH-O-M$  are preferably prepared according to the synthetic routes outlined in Schemes 6-8.

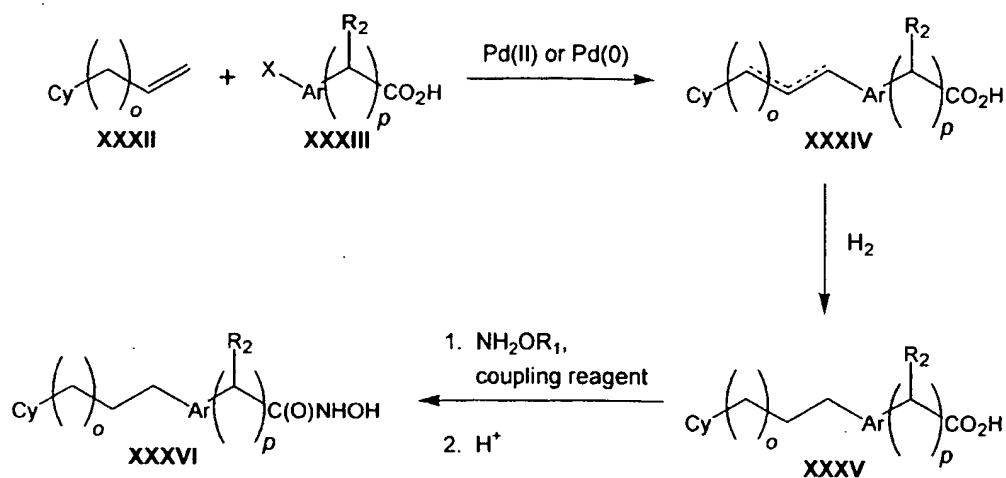
Accordingly, in certain preferred embodiments, compounds of formulae XXIX and XXXI ( $L^2 = -C(O)-CH=CH-$  or  $-C(O)-CH_2CH_2-$ ) preferably are prepared according to the route described in Scheme 6. Thus, a substituted

10 aryl acetophenone (XXV) is treated with an aryl aldehyde (XXVI) in a protic solvent such as methanol in the presence of a base such as sodium methoxide to afford the enone XXVII.

The acid substituent of XXVII ( $R = H$ ) is coupled with an *O*-protected hydroxylamine such as *O*-tetrahydropyranylhdroxylamine ( $R_1 =$  tetrahydropyranyl) to yield the *O*-protected-*N*-hydroxybenzamide XXVIII. The coupling reaction is preferably performed by treating the acid and  
5 hydroxylamine with dicyclohexylcarbodiimide in a solvent such as methylene chloride or with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in the presence of *N*-hydroxybenzotriazole in a solvent such as dimethylformamide. Other coupling reagents are known in the art and may also be used in this reaction. *O*-Deprotection is accomplished by treatment of XXVIII with an  
10 acid such as camphorsulfonic acid in a solvent such as methanol to afford the hydroxamic acid XXIX ( $L^2 = -C(O)-CH=CH-$ ).

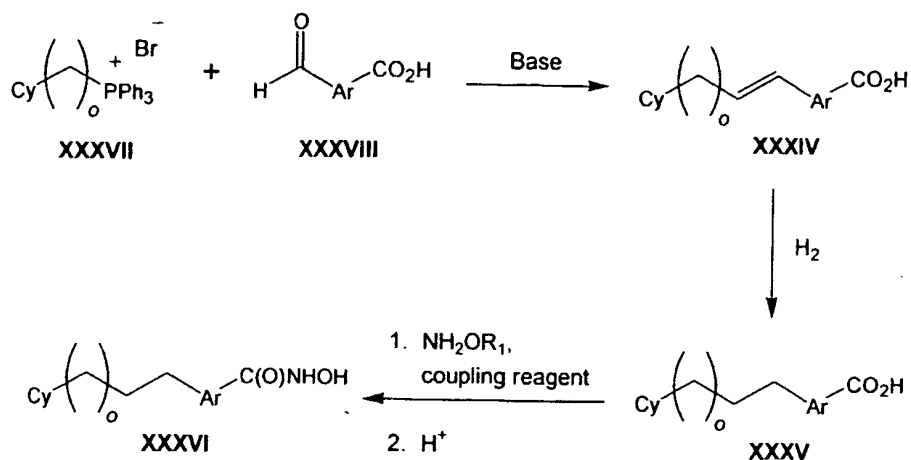
Saturated compounds of formula XXXI ( $L^2 = -C(O)-CH_2CH_2-$ ) are preferably prepared by hydrogenation of XXVII ( $R = Me$ ) over a palladium catalyst, such as 10% Pd/C, in a solvent such as methanol-tetrahydrofuran.  
15 Basic hydrolysis of the resulting saturated ester XXX with lithium hydroxide, followed by *N*-hydroxy amide formation and acid hydrolysis as described above, then yields the hydroxamic acid XXXI.

Scheme 7



- 5 Compounds of formula XXXVI ( $L^2 = -(\text{CH}_2)_{o+2}-$ ) are preferably prepared by the general procedures described in Scheme 7. Thus, in some embodiments, a terminal olefin (XXXII) is coupled with an aryl halide (XXXIII) in the presence of a catalytic amount of a palladium source, such as palladium acetate or tris(dibenzylideneacetone)dipalladium(0), a phosphine,
- 10 such as triphenylphosphine, and a base, such as triethylamine, in a solvent such as acetonitrile to afford the coupled product XXXIV. Hydrogenation, followed by *N*-hydroxyamide formation and acid hydrolysis, as described above, yields the hydroxamic acid XXXVI.

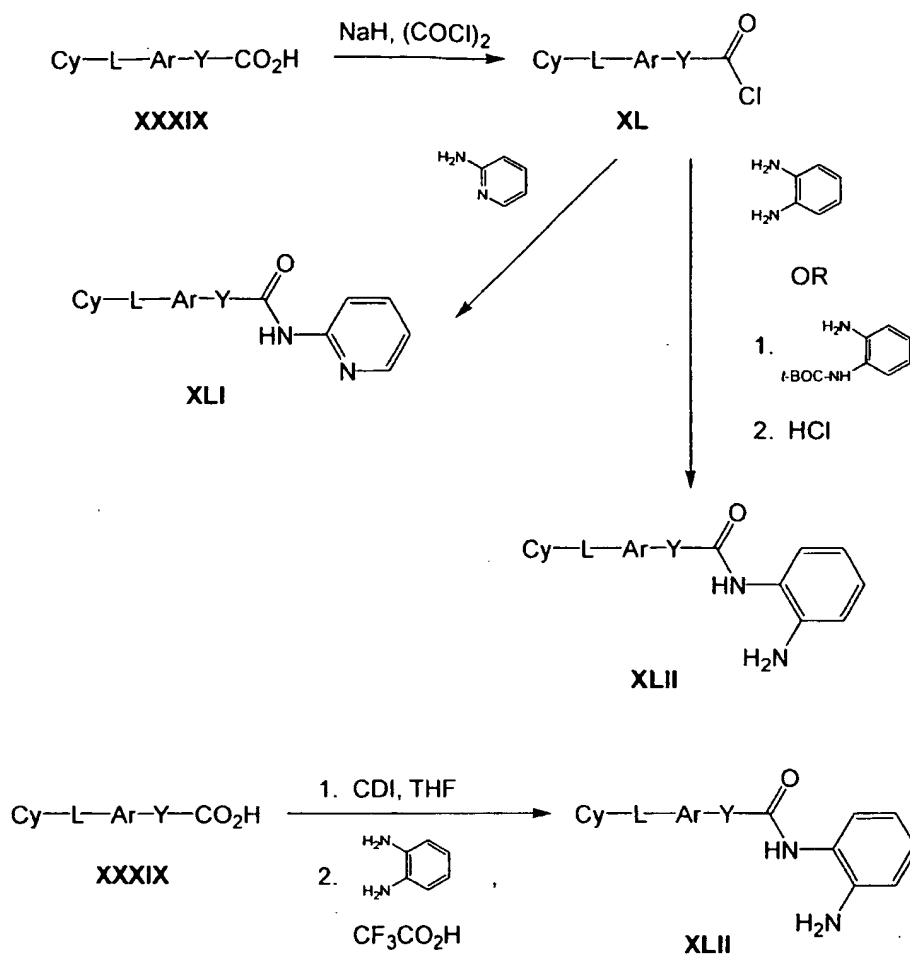
Scheme 8



Alternatively, in some other embodiments, a phosphonium salt of formula XXXVII is treated with an aryl aldehyde of formula XXXVIII in the presence of base, such as lithium hexamethyldisilazide, in a solvent, such as tetrahydrofuran, to produce the compound XXXIV. Hydrogenation, followed by *N*-hydroxyamide formation and acidic hydrolysis, then yields the compounds XXXVI (Scheme 8).

Compounds of formula Cy-L-Ar-Y-C(O)-NH-Z, wherein L is L<sup>1</sup> or L<sup>2</sup>, as previously described herein, Y is Y<sup>1</sup> or Y<sup>2</sup>, as previously described herein, and Z is anilinyll or pyridyl or thiadiazolyl, are preferably prepared according to synthetic routes outlined in Scheme 9.

Scheme 9

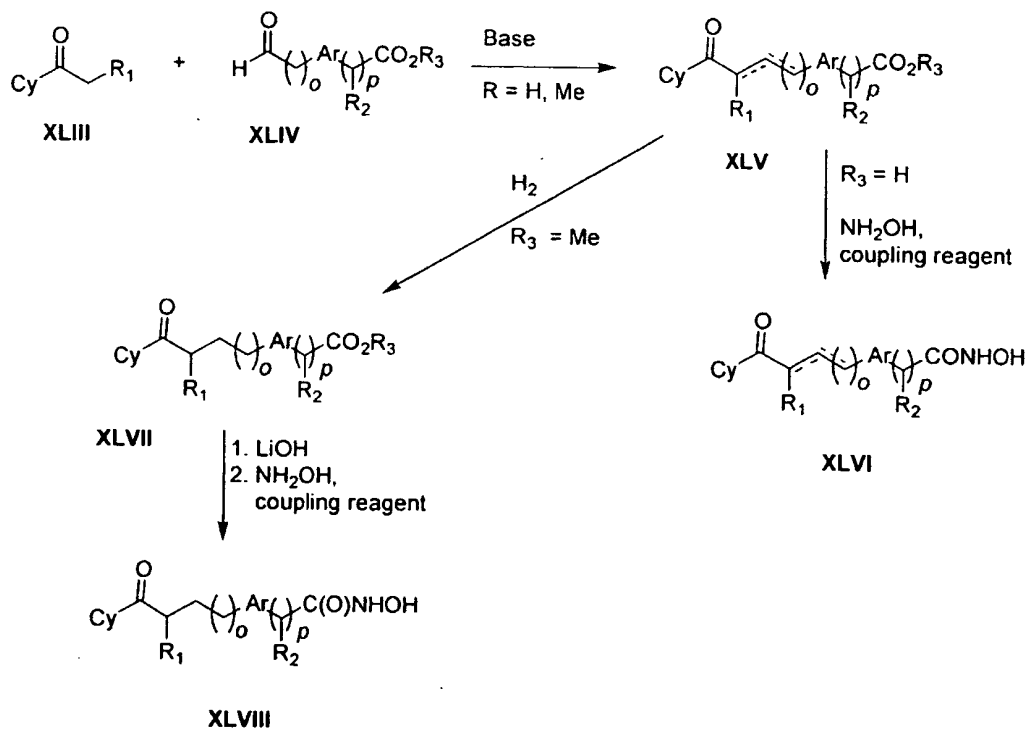


An acid of formula  $\text{Cy-L-Ar-Y-C(O)-OH}$  (**XXXIX**), prepared by one of the methods shown in Schemes 4-8, is converted to the corresponding acid chloride **XL** according to standard methods, e.g., by treatment with sodium hydride and oxalyl chloride. Treatment of **XL** with 2-aminopyridine and a tertiary base such as *N*-methylmorpholine, preferably in dichloromethane at

- reduced temperature, then yields the pyridyl amide XLI. In a similar fashion, the acid chloride XL may be treated with 1,2-phenylenediamine to afford the anilinyll amide XLII. Alternatively, the acid chloride XL may be treated with a mono-protected 1,2-phenylenediamine, such as 2-(*t*-BOC-amino)aniline, followed by deprotection, to yield XLII.

In an alternative procedure, the acid XXXIX may be activated by treatment with carbonyldiimidazole (CDI), followed by treatment with 1,2-phenylenediamine and trifluoroacetic acid to afford the anilinyll amide XLII.

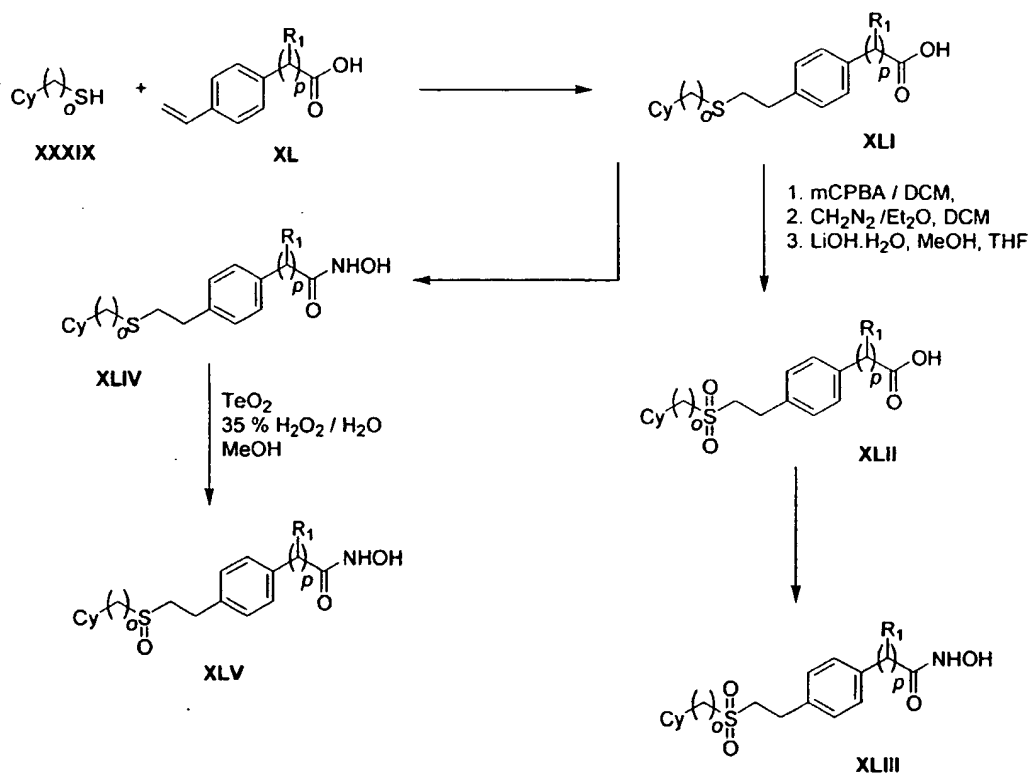
Scheme 10





Compounds of formula XLVIII ( $L^2 = -C(O)-alkylene-$ ) preferably are prepared according to the general procedure depicted in Scheme 10. Thus, Aldol condensation of ketone XLIII ( $R_1 = H$  or alkyl) with aldehyde XLIV affords the adduct XLV. The adduct XLV may be directly converted to the corresponding hydroxamic acid XLVI. Hydrogenation of XLV may yield the saturated compound XLVII and which is then converted to the hydroxamic acid XLVIII.

Scheme 11



Compounds of formula (5), wherein one of the carbon atoms in L<sup>2</sup> is replaced with S, S(O), or S(O)<sub>2</sub> preferably are prepared according to the general procedure outlined in Scheme 11. Thus, thiol XLIX is added to olefin L to produce LI. The reaction is preferably conducted in the presence of a radical initiator such as 2,2'-azobisisobutyronitrile (AIBN) or 1,1'-azobis(cyclohexanecarbonitrile) (VAZO™). Sulfide oxidation, preferably by treatment with *m*-chloroperbenzoic acid (mCPBA), affords the corresponding sulfone, which is conveniently isolated after conversion to the methyl ester by treatment with diazomethane. Ester hydrolysis then affords the acid LII, which is converted to the hydroxamic acid LIII according to any of the procedures described above. The sulfide LI also may be converted directly to the corresponding hydroxamic acid LIV, which then may be selectively oxidized to the sulfoxide LV, for example, by treatment with hydrogen peroxide and tellurium dioxide.

The reagents according to the invention are useful as analytical tools and as therapeutic tools, including gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

The invention also provides method for inhibiting HDAC-4 activity in a cell, comprising contacting the cell with a specific inhibitor of HDAC-4, whereby HDAC-4 activity is inhibited. As used herein, the term "specific inhibitor" means any molecule or compound that decreases the amount of HDAC RNA, HDAC protein, and/or HDAC protein activity in a cell. Particularly preferred specific inhibitors decrease the amount of RNA, protein, and/or protein activity in a cell for HDAC-1 and/or HDAC-4.

In an embodiment thereof, the invention provides a method for inhibiting the HDAC-4 isoform in a cell comprising contacting the cell with an

antisense oligonucleotide of the first aspect of the invention. Preferably, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the invention further comprises contacting the cell with HDAC-4 small molecule inhibitor that interacts with and reduces the enzymatic activity of the HDAC-4 isoform. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

Thus, the antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases, including benign and malignant neoplasms, by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of HDAC-4 antisense oligonucleotide or a small molecule HDAC-4 inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted.

An assessment of cell proliferation can be made by counting cells that have been contacted with the oligonucleotide or small molecule of the invention and compare that number with the number of non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a hemacytometer. Where the cells are in a solid growth (e.g., a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth of the tissue or organ with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells. More preferably, the term includes a retardation of cell proliferation that is

100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, HDAC-4 antisense oligonucleotide or HDAC-4 small molecule inhibitor that inhibits  
5 cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, growth arrest, programmed cell death (*i.e.*, to apoptose), or necrotic cell death.

The cell proliferation inhibiting ability of the antisense oligonucleotides according to the invention allows the synchronization of a population of a-  
10 synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization  
15 of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection  
20 efficiency.

The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.

In yet other preferred embodiments, the cell contacted with HDAC-4 antisense oligonucleotide is also contacted with HDAC-4 small molecule  
25 inhibitor.

As used herein, the term "histone deacetylase small molecule inhibitor" denotes an active moiety capable of interacting with one or more specific histone deacetylase isoforms at the protein level and reducing the activity of that histone deacetylase isoform. Particularly preferred are histone deacetylase small molecule inhibitors that inhibit the HDAC-1 and/or the HDAC-4 isoform. An HDAC-1 small molecule inhibitor is a molecule that reduces the activity of the HDAC-1 isoform. An HDAC-4 small molecule inhibitor is a molecule that reduces the activity of the HDAC-4 isoform. In a preferred embodiment, the reduction of activity is at least 5-fold, more preferably at least 10-fold, most preferably at least 50-fold. In another embodiment, the activity of the histone deacetylase isoform is reduced 100-fold. As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of HDAC-4 and/or the HDAC-1 isoform that is involved in tumorigenesis.

In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated with well known pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.

The term "operably associated with" or "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small

molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule inhibitor. In some preferred embodiments, an antisense oligonucleotide of  
5 the invention that targets one particular histone deacetylase isoform (*e.g.*, HDAC-4) is operably associated with an small molecule inhibitor which targets the same histone deacetylase isoform (*e.g.*, HDAC-4). A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable association is a covalent linkage between the antisense oligonucleotide and  
10 the histone deacetylase small molecule inhibitor. Such a covalent linkage is hydrolyzable, for example, by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

In certain preferred embodiments, the covalent linkage may be directly  
15 between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone of the oligonucleotide. Alternatively, the covalent linkage may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase  
20 small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide, a lipid or a glycolipid. Another useful operable associations include lipophilic association, such as the formation of a liposome containing an antisense oligonucleotide and the  
25 histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule. Such lipophilic molecules include, without limitation, phosphatidylcholine, cholesterol, phosphatidylethanolamine, and synthetic

neoglycolipids, such as sialyllacNAc-HDPE. In certain preferred  
embodiments, the operable association may not be a physical association, but  
simply a simultaneous co-existence in the body, for example, when the  
antisense oligonucleotide is associated with one liposome and the small  
5 molecule inhibitor is associated with another liposome.

In a third aspect, the invention provides a method for inhibiting  
neoplastic cell proliferation in an animal, comprising administering to an  
animal having at least one neoplastic cell present in its body a therapeutically  
effective amount of a specific inhibitor of HDAC-4, whereby neoplastic cell  
10 proliferation is inhibited in the animal. In an embodiment thereof, the  
invention provides a method for inhibiting neoplastic cell growth in an  
animal. In this method, a therapeutically effective amount of the antisense  
oligonucleotide of the invention is administered to an animal having at least  
one neoplastic cell present in its body, the oligonucleotide being administered  
15 with a pharmaceutically acceptable carrier for a therapeutically effective  
period of time. Preferably, the animal is a mammal, particularly a  
domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant  
cell growth. A neoplastic cell may be a hyperplastic cell, a cell that shows a  
20 lack of contact inhibition of growth *in vitro*, a benign tumor cell that is  
incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis"  
is used to denote the induction of uncharacteristic or untimely cell  
proliferation that leads to the development of a neoplastic growth.

25 As used herein, the term "therapeutically effective amount" means the  
total amount of each active component of the pharmaceutical composition or  
method that is sufficient to show a meaningful patient benefit, *i.e.*, inhibiting

HDAC activity, particularly HDAC-1 and/or HDAC-4 activity or to inhibit neoplastic growth or for the treatment of proliferative diseases and disorders.

When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term  
5 refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

Administration of the synthetic oligonucleotide of the invention used in the pharmaceutical composition or to practice the method of the present  
10 invention can be carried out in a variety of conventional ways, such as intraocular, oral ingestion, inhalation, or cutaneous, subcutaneous, intramuscular, or intravenous injection.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered orally, the synthetic oligonucleotide will be in  
15 the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% synthetic oligonucleotide and preferably from about 25 to 90% synthetic  
20 oligonucleotide. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene  
25 glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by



weight of the synthetic oligonucleotide and preferably from about 1 to 50% synthetic oligonucleotide.

When a therapeutically effective amount of synthetic oligonucleotide of the invention is administered by intravenous, subcutaneous, intramuscular, 5 intraocular, or intraperitoneal injection, the synthetic oligonucleotide will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, subcutaneous, intramuscular, 10 intraperitoneal, or intraocular injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also 15 contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments 20 which the patient has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the 25 optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present

invention should contain about 10  $\mu\text{g}$  to about 20 mg of synthetic oligonucleotide per kg body or organ weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01  $\mu\text{M}$  to about 20  $\mu\text{M}$ . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05  $\mu\text{M}$  to about 15  $\mu\text{M}$ . In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1  $\mu\text{M}$  to about 10  $\mu\text{M}$ .

For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective amount of HDAC-4 antisense oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.

The method may further comprise administering to the animal a therapeutically effective amount of an HDAC-4 small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time. In some preferred embodiments, the histone deacetylase small molecule  
5 inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule  
10 inhibitor from about 0.01  $\mu\text{M}$  to about 10  $\mu\text{M}$ . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05  $\mu\text{M}$  to about 10  $\mu\text{M}$ . In a more preferred embodiment, the  
15 blood level of histone deacetylase small molecule inhibitor is from about 0.1  $\mu\text{M}$  to about 5  $\mu\text{M}$ . For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg  
protein effector per kg body weight per day. In a more preferred  
embodiment, a total dosage of histone deacetylase small molecule inhibitor  
20 will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 25  
mg protein effector per kg body weight per day. In a particularly preferred  
embodiment, the therapeutically effective synergistic amount of histone  
25 deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide) is about 5 mg per kg body weight per day.

When the method of the invention results in an improved inhibitory effect, the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*, histone deacetylase small molecule inhibitor) required to  
5 obtain a given inhibitory effect are reduced as compared to those necessary when either is used individually.

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning and  
10 altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example empirically by starting at relatively low amounts and by step-wise increments  
15 with concurrent evaluation of inhibition.

In a fourth aspect, the invention provides a method for inhibiting HDAC-4 isoform in a cell comprising contacting the cell with a small molecule inhibitor of the first aspect of the invention. In certain preferred embodiments of the fourth aspect of the invention, cell proliferation is  
20 inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth.

In a fifth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal  
25 having at least one neoplastic cell present in its body a therapeutically effective amount of a small molecule inhibitor of the first aspect of the

invention with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01  $\mu$  M to about 10  $\mu$  M. In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05  $\mu$  M to about 10  $\mu$  M. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about 0.1  $\mu$  M to about 5  $\mu$  M. For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor ranges from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor ranges from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 25 mg protein effector per kg body weight per day.

In a sixth aspect, the invention provides a method of inhibiting the induction of cell proliferation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of HDAC-4 or contacting a cell with a small molecule inhibitor of HDAC-4. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis.

The invention further provides for histone deacetylase small molecule inhibitors that may be generated which specifically inhibit the histone

deacetylase isoform(s) required for inducing cell proliferation, *e.g.*, HDAC-1 and HDAC-4, while not inhibiting other histone deacetylase isoforms not required for inducing cell proliferation. Accordingly, in a seventh aspect, the invention provides a method for identifying a small molecule histone

5 deacetylase inhibitor that inhibits the HDAC-4 isoform and or the HDAC-1 isoform, which is required for the induction of cell proliferation. The method comprises contacting the HDAC-4 and/or the HDAC-1 isoform with a candidate small molecule inhibitor and measuring the enzymatic activity of the contacted histone deacetylase isoform, wherein a reduction in the

10 enzymatic activity of the contacted histone deacetylase isoform identifies the candidate small molecule inhibitor as a small molecule histone deacetylase inhibitor that inhibits the histone deacetylase isoform, *i.e.*, HDAC-4 and/or HDAC-1.

Measurement of the enzymatic activity of HDAC-4 or HDAC-1 may be

15 achieved using known methodologies. For example, Yoshida *et al.* (*J. Biol. Chem.* 265:17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in trichostatin A treated cells. Taunton *et al.* (*Science* 272:408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous

20 and recombinant HDAC. Both Yoshida *et al.* (*J. Biol. Chem.* 265:17174-17179, 1990) and Taunton *et al.* (*Science* 272:408-411, 1996) are hereby incorporated by reference.

Preferably, the histone deacetylase small molecule inhibitor that inhibits the HDAC-4 and or the HDAC-1 isoform required for induction of

25 cell proliferation is an HDAC-4 small molecule inhibitor that interacts with and reduces the enzymatic activity of the HDAC-4 and/or the HDAC-1 isoform.

In an eighth aspect, the invention provides a method for identifying a small molecule histone deacetylase inhibitor that inhibits the HDAC-4 isoform involved in the induction of cell proliferation. The method comprises contacting a cell with a candidate small molecule inhibitor and measuring the enzymatic activity of the contacted histone deacetylase isoform, wherein a reduction in the enzymatic activity of the HDAC-4 isoform identifies the candidate small molecule inhibitor as a small molecule histone deacetylase inhibitor that inhibits HDAC-4.

In a ninth aspect, the invention provides a small molecule histone deacetylase inhibitor identified by the method of the seventh or the eighth aspects of the invention. Preferably, the histone deacetylase small molecule inhibitor is substantially pure.

In a tenth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits expression of HDAC-4 isoform, a small molecule histone deacetylase inhibitor that inhibits expression or activity of HDAC-4 isoform, an antisense oligonucleotide that inhibits expression of the HDAC-1 isoform, a small molecule histone deacetylase inhibitor that inhibits the expression or the activity of the HDAC-1 isoform, an antisense oligonucleotide that inhibits expression of a DNA methyltransferase, and a small molecule DNA methyltransferase inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

In an eleventh aspect, the invention provides a method of inhibiting neoplastic cell growth comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits expression of HDAC-4 isoform, a small molecule histone deacetylase inhibitor that inhibits the expression or the activity of HDAC-4 isoform, an antisense oligonucleotide that inhibits expression of the HDAC-1 isoform, a small molecule histone deacetylase inhibitor that inhibits expression or activity of the HDAC-1 isoform, an antisense oligonucleotide that inhibits expression of a DNA methyltransferase, and a small molecule DNA methyltransferase inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

Antisense oligonucleotides that inhibit DNA methyltransferase are described in Szyf and von Hofe, U.S. Patent No. 5,578,716. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.



### EXAMPLES

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than  
5 routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the appended claims.

#### **Example 1**

##### Synthesis and Identification of Antisense Oligonucleotides

10 Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC. All oligos used  
15 were 20 base pairs in length.

To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were  
20 initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS as an ODN with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base mismatch.

25 Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank

Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

5           Twenty-one phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3. HDAC-3 MM oligo was created as a control; compared to the antisense oligo, it contains a 6-  
10   base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS was identified as an ODN with antisense activity to human HDAC-4. HDAC-4 MM oligo  
15   was created as a control; compared to the antisense oligo, it contains a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-6 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6  
20   AS was identified as an ODN with antisense activity to human HDAC-6. HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

## Example 2

25    HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, Human A549 cells were treated with 50 nM of antisense (AS) oligo directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or  
5 100 nM of AS oligo directed against human HDAC-4 or its MM oligo (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC)  
10 (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to  
15 the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

Cells were harvested, and total RNAs were analyzed by Northern blot  
20 analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty µg of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was  
25 performed using conventional procedures.

As presented in Figures 3A and 3B, respectively, the expression of HDAC-3 mRNA and HDAC-4 mRNA in human A549 cells was inhibited by treatment

with the respective antisense oligonucleotides. These results indicate that HDAC ASODNs can specifically inhibit targeted HDAC expression at the mRNA level.

### Example 3

#### HDAC OSDNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC OSDNs would inhibit HDAC protein expression, human A549 cancer cells were treated with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. OSDN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X- 100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, plus protease inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 ug of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. *et al.*, (1999) *Mol. Cell. Biol.* 19:7816-27), and was used at a dilution of 1:1000. Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform. HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 4, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein.

5 In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are  
10 overexpressed in cancer cell lines.

**Table 3: Expression Level of HDAC Isotypes in  
Human  
Normal and Cancer Cells**

<u>Normal or Cancer</u>	<u>Tissue Type</u>	<u>Cell Designation</u>	<u>HDAC-6</u>	<u>HDAC-2</u>	<u>HDAC-1</u>	<u>HDAC-3</u>	<u>HDAC-4</u>	<u>HDAC-5</u>	<u>HDAC-7</u>
Normal	Breast Epithelial	HMEC	+	+	-	++	+	-	-
Normal	Foreskin Fibroblasts	MRHF	+	+	-	+	++	-	++
Cancer	Bladder	T24	+++	++	+++	+++	++	+	++
Cancer	Lung	A549	++	+++	++	+++	+++	+++	+
Cancer	Colon	SW48	+++	+++	+++	+++	+++		
Cancer	Colon	HCT116	+++	+++	++++	+++	++++	+	-
Cancer	Colon	HT29	+++	+++	+++	+++	+++		
Cancer	Colon	NCI-H446	++	++++	++	+++	++++	++++	++
Cancer	Cervix	Hela	+++	++++	+++	+++	+++		
Cancer	Prostate	DU145	+++	+++	+++	+++	++++		

Cancer	Breast	MDA-MB-231	++++	+++	++	+++	+++	
Cancer	Breast	MCF-7	++	+++	+++	+++	++	
Cancer	Breast	T47D	+++	+++	+++	+++	++	
Cancer	Kidney	293T	++	++++	+++	++++	++	++++
Cancer	Leukemia	K562	++++	++++	+++	++++	++++	
Cancer	Leukemia	Jurkat T	++	++	+++	++++	++	++

(-): not detectable; (+): detectable; (++) : 2X over (+); (+++): 5X over (+); (++++): 10X over (+)

#### Example 4

##### Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis

5 In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAMB231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

10 For the apoptosis study, cells were analyzed using the Cell Death Detection ELISA <sup>Plus</sup> kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated



in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at 410 nm. The reference was set at 490 nm.

- 5 For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a hemocytometer. Percentage of inhibition was calculated as (100 - AS cell numbers/control cell  
10 numbers)%.

Results of the study are shown in Figure 5 and Figure 6, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human cancer cells (Figure 5 and Figure 6, Table 4 and Table 5). The corresponding  
15 mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and treatment with human HDAC-2 OSDN has a  
20 minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells are p53 wild type, this induction of apoptosis is independent of p53 activity.

**Table 4: Gene Transcription Altered by HDAC-4 AS1**

gene name	fold change
CDK4	-3
cyclin A2	-3
cyclin B1	-3
p21	4
PLK	-4

topo II $\alpha$	-5
GADD153	6
GADD45	3
Notch-4	-3
basic FGF	2
Egr-1	3
IL-15	4
IRF	2

Human A549 cells were treated with 50 nM oligos for two days before total RNAs were harvested for cDNA array analysis;

Fold change on transcriptions was compared to that of HDAC-4 mismatch oligo (MM2) treated cells;

Expression of 39 genes altered by HDAC-4 AS1 out of 588 genes

**Table 5**

**Effect of HDAC Isotype-Specific OSDNs on Human Normal and Cancer Cells Apoptosis After 48 Hour Treatment**

	A549	T24	MDAmb231	HMEC
HDAC-1	+	-		-
HDAC-2	-	-	-	-
HDAC-3	-	-	-	-
HDAC-4	+++	+	++	-
HDAC-6	-	-	-	-
TSA (100ng/ml)	++	++	++	+

5

"-":  $\leq 2$ x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold;

"+++": 5-8X fold; "++++": 8X fold

10

### Example 5

### Inhibition of HDAC Isotypes Induces the Expression of Growth Regulatory Genes

In order to understand the mechanism of growth arrest and apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment, RNase

- 5 protection assays were used to analyze the mRNA expression of cell growth regulators (p21 and *GADD45*) and proapoptotic gene *Bax*.

- Briefly, human cancer A549 or T24 cells were treated with HDAC isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total RNAs were extracted and RNase protection assays were performed to
- 10 analyzed the mRNA expression level of p21 and *GADD45*. As a control, A549 cells were treated by lipofectin with or without TSA (250 ng/ml) treatment for 16 hours. These RNase protection assays were done according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled
- 15 probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San Diego, California, U.S.A.) according to the manufacturer's instructions. Protection procedures were performed using "RPA II™ Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation
- 20 of the bands from autoradiograms was done by using Cyclone™ Phosphor System (Packard Instruments Co. Inc., Meriden, CT). The results are shown in Figure 7 and Table 6.

Table 6

Up-Regulation of p21, *GADD45* and *Bax* After Cell  
Treatment with Human HDAC Isotype-Specific Antisenses

	A549			T24		
	p21	<i>GADD45</i>	<i>Bax</i>	p21	<i>GADD45</i>	<i>Bax</i>
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

- 5 Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

As can be seen in Figure 7, the inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression.

- 10 Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45* expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8  $\mu$ M, 16 hrs).

- 15 Experiments were also conducted to examine the affect of HDAC antisense oligonucleotides on HDAC protein expression. In A549 cells,

treatment with HDAC-4 antisense oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 8).

#### Example 7

##### 5                    Inhibition of HDAC Isotypes by Small Molecules

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned  
10 recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozinger, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 96:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human  
15 embryonic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium precipitates, formed by  
20 mixing DNA, calcium chloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM

medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

- HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

- For the deacetylase assay, 20,000 cpm of an [ $^3\text{H}$ ]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37°C. For the HDAC-1 assay, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [ $^3\text{H}$ ]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 40°C prior to the start of the enzymatic assay. IC<sub>50</sub> values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

### Example 8

#### Inhibition of HDAC Activity in Whole Cells by Small Molecules

T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were  
5 extracted from the cells by standard procedures (see *e.g.* Yoshida *et al.*, *supra*) after the culture period. Twenty µg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, WY). Horse Radish Peroxidase conjugated  
10 secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

15 The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

### Example 9

#### Inhibition of Cancer Growth by HDAC Small Molecule Inhibitors

Four thousand five hundred (4,500) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5  
diphenyl tetrazolium bromide) assay to quantitatively determine cell  
proliferation and cytotoxicity. Typically, HCT116 cells were plated into each  
25 well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations (1 µM, 5 µM and 25 µM, in DMSO)

were added in triplicate into the culture media (final DMSO concentration 1%) and incubated for 48 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO<sub>2</sub>. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubate for overnight at 37°C in incubator with 5% CO<sub>2</sub>. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM.

The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

#### Example 10

##### Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145,  $2 \times 10^6$ ) or human colon cancer cells (HCT116;  $2 \times 10^6$ ) or small lung core A549  $2 \times 10^6$  were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperitoneal or oral administration was initiated when the tumors reached a volume of 100 mm<sup>3</sup>. For intraperitoneal administration, small molecule inhibitors of HDAC (40–50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50mg/kg body weight/days) were dissolved in a solution containing 65% polyethylene



glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from  
5 vehicle-treated mice as controls.

The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to  
5 be encompassed by the following claims.

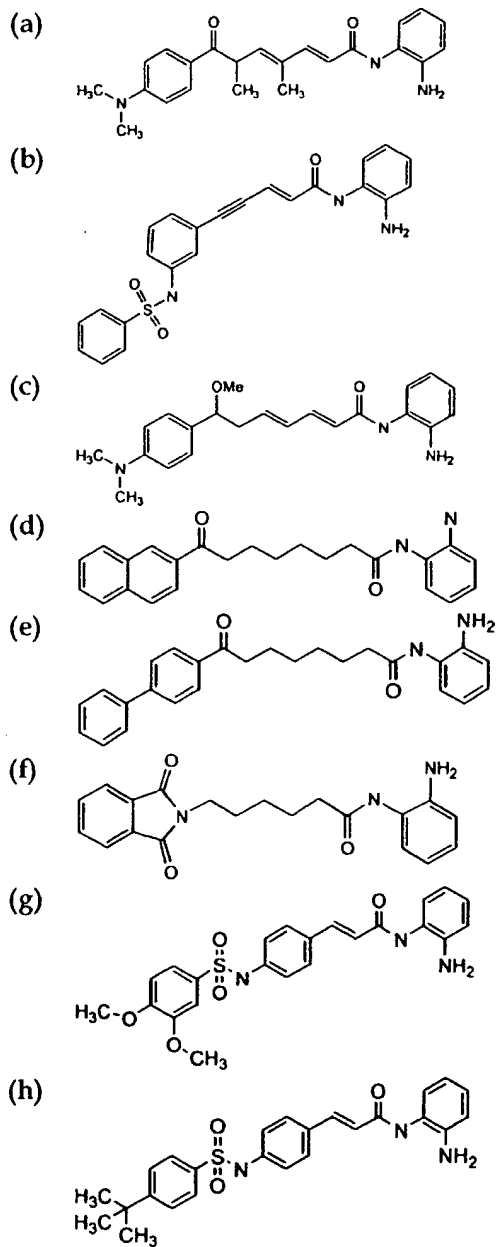
What is claimed is:

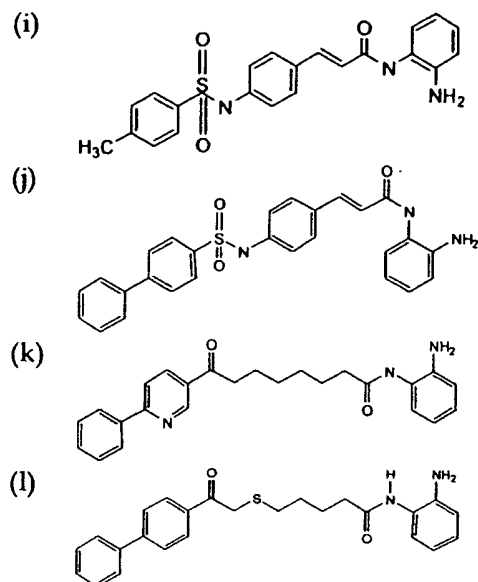
- 5     1.     A method of inhibiting HDAC-4 activity in a cell, comprising contacting the cell with an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4, whereby HDAC-4 activity is inhibited.
- 10    2.     The method according to claim 1, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is a chimeric oligonucleotide.
3.     The method according to claim 1, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is a hybrid oligonucleotide.
- 15    4.     The method according to claim 1, wherein the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.
- 20    5.     The method according to claim 1, wherein the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

25

6. The method according to claim 1, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11.
7. The method according to claim 1, whereby inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell.
8. The method according to claim 1, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell.
9. The method according to claim 1, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell.
10. The method according to claim 1, wherein inhibition of HDAC-4 activity in the contacted cell further leads to programmed cell death of the contacted cell.
11. The method according to claim 8, wherein inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the contacted cell.
12. A method of inhibiting HDAC-4 activity in a cell, comprising contacting the cell with a small molecule inhibitor of HDAC-4 selected from

the group consisting of:





13. The method according to claim 12, whereby inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell.

5

14. The method according to claim 12, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell.

10 15. The method according to claim 12, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell.

16. The method according to claim 12, wherein inhibition of HDAC-4

activity in the contacted cell further leads to programmed cell death of the contacted cell.

17. The method according to claim 13, wherein inhibition of HDAC-4  
5 activity in the contacted cell further leads to necrotic cell death of the contacted cell.

18. A method for inhibiting neoplastic cell proliferation in an animal,  
comprising administering to an animal having at least one neoplastic cell  
10 present in its body a therapeutically effective amount of an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4, whereby neoplastic cell proliferation is inhibited.

19. The method according to claim 18, wherein the animal is  
15 administered a chimeric HDAC-4 antisense oligonucleotide.

20. The method according to claim 18, wherein the animal is  
administered a hybrid HDAC-4 antisense oligonucleotide.

20

21. The method according to claim 18, wherein the antisense  
oligonucleotide has a nucleotide sequence of from about 13 to about 35  
nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

22. The method according to claim 18, wherein the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.
- 5 23. The method according to claim 18, wherein the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.
- 10 24. The method according to claim 18, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11.
25. The method according to claim 18, whereby inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell.
- 15 26. The method according to claim 18, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell.
- 20 27. The method according to claim 18, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell.
- 25 28. The method according to claim 18, wherein inhibition of HDAC-4 activity in the contacted cell further leads to programmed cell death of the



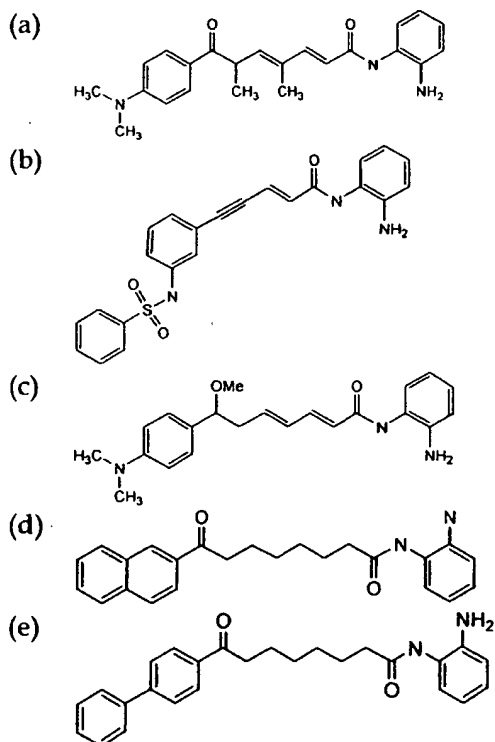
contacted cell.

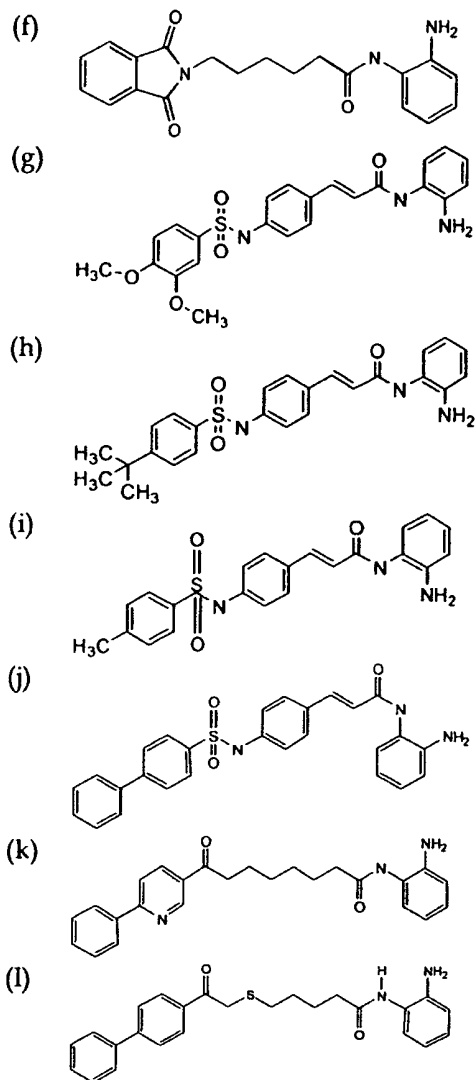
29. The method according to claim 25, wherein inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the

5 contacted cell.

30. A method for inhibiting neoplastic cell proliferation in an animal, comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of a small molecule

10 inhibitor selected from the group consisting of:





31. The method according to claim 30, whereby inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell.

32. The method according to claim 30, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell.
- 5 33. The method according to claim 30, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell.
- 10 34. The method according to claim 30, wherein inhibition of HDAC-4 activity in the contacted cell further leads to programmed cell death of the contacted cell.
- 15 35. The method according to claim 31, wherein inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the contacted cell.
36. The method according to claim 18 or 30, wherein the animal is a human.
- 20 37. The method according to claim 18 or 30, further comprising administering to an animal a therapeutically effective amount of an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-1.

38. The method according to claim 37, wherein the animal is administered a chimeric HDAC-1 antisense oligonucleotide.
- 5 39. The method according to claim 37, wherein the animal is administered a hybrid HDAC-1 antisense oligonucleotide.
- 10 40. The method according to claim 37, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.
- 15 41. The method according to claim 37, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.
- 20 42. The method according to claim 37, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.
- 25 43. The method according to claim 37, wherein the animal is administered an HDAC-1 antisense oligonucleotide that is SEQ ID NO:5.
44. A composition comprising an agent that specifically inhibits the

activity of HDAC-4.

45. The composition according to claim 1, wherein the agent is an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4.

46. The composition according to claim 2, wherein the antisense oligonucleotide is a chimeric oligonucleotide.

47. The composition according to claim 2, wherein the antisense oligonucleotide is a hybrid oligonucleotide.

48. The composition according to claim 2, wherein the antisense oligonucleotide has a nucleotide sequence of from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

49. The composition according to claim 2, wherein the antisense oligonucleotide has a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

50. The composition according to claim 2, wherein the antisense oligonucleotide has a nucleotide sequence of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

51. The composition according to claim 2, wherein the antisense

oligonucleotide is SEQ ID NO:11.

52. The composition according to claim 2, wherein the antisense oligonucleotide has one or more phosphorothioate internucleoside linkages.

5

53. The composition according to claim 9, wherein the antisense oligonucleotide further comprises a length of 20-26 nucleotides.

54. The composition according to claim 10, wherein the oligonucleotide is modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2' -O- methyl groups attached to their sugar residues.

10

55. The composition according to claim 1, wherein the agent is a small molecule inhibitor of HDAC-4.

15

56. The composition according to claim 12, wherein the structure of the small molecule inhibitor is selected from the group consisting of:

20 (a)  $\text{Cy-CH(OMe)-Y}^1\text{-C(O)-NH-Z}$  (1)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S;

25

S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted;

5 (b) Cy-Y<sup>2</sup>-C(O)-NH-Z (2)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocycl, any of which may be optionally substituted; Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is aniliny or pyridyl, or thiadiazolyl, any of which may be optionally substituted;

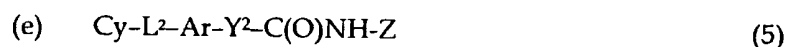
(c) Cy-B-Y<sup>3</sup>-C(O)-NH-Z  
(3)

15 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocycl, any of which may be optionally substituted; B is selected from the group consisting of -CH(OMe), ketone and methylene; Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted;

25 (d) Cy-L<sup>1</sup>-Ar-Y<sup>1</sup>-C(O)-NH-Z (4)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocycl, any of

which may be optionally substituted; L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, wherein said alkylene may be optionally substituted; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when L<sup>1</sup> is -(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>-, n being 1, 2, or 3, and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indoliny;

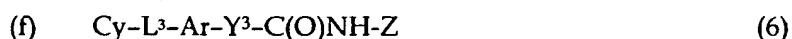


wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be

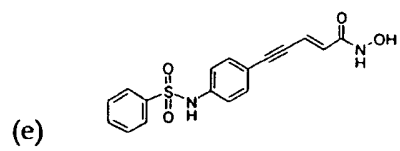
optionally substituted, provided that Cy is not a spirocycloalkyl)heterocyclyl; L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>2</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, which



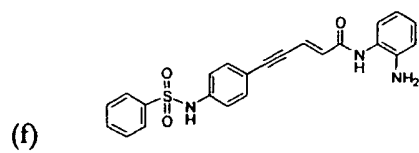
may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula  $-C(O)R$  wherein R comprises an  $\alpha$ -amino acyl moiety; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and  $-O-M$ , M being H or a pharmaceutically acceptable cation; provided that when the carbon atom to which Cy is attached is oxo substituted, then Cy and Z are not both pyridyl;



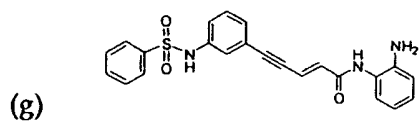
wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a spirocycloalkyl)heterocyclyl;  $L^3$  is selected from the group consisting of (a)  $-(CH_2)_m\text{-W-}$ , where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of  $-C(O)NH-$ ,  $-S(O)_2NH-$ ,  $-NHC(O)-$ ,  $-NHS(O)_2-$ , and  $-NH-C(O)-NH-$ ; and (b)  $C_1\text{-C}_6$  alkylene or  $C_2\text{-C}_6$  alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that  $L^3$  is not  $-C(O)-$ , and wherein one of the carbon atoms of the alkylene optionally may be replaced by O;  $NR'$ ,  $R'$  being alkyl, acyl, or hydrogen; S;  $S(O)$ ; or  $S(O)_2$ ; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and  $Y^3$  is  $C_2$  alkenylene or  $C_2$  alkynylene, wherein one or both carbon atoms of the alkenylene optionally may be substituted with alkyl, aryl, alkaryl, or aralkyl; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and  $-O-M$ , M being H or a pharmaceutically acceptable cation; provided that when Cy is unsubstituted phenyl, Ar is not phenyl wherein  $L^3$  and  $Y^3$  are oriented *ortho* or *meta* to each other;



(7)

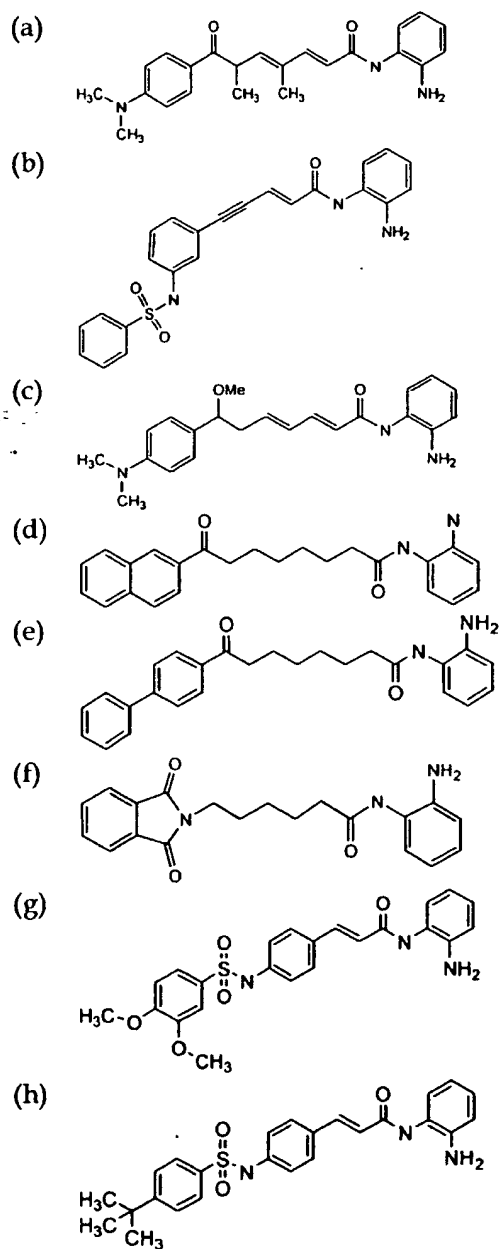


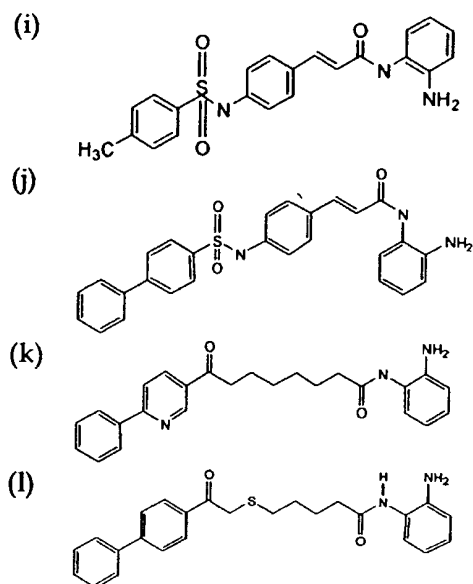
(8) and



(9).

57. The composition according to claim 13, wherein the small molecule inhibitor is selected from the group consisting of:





58. A method for inhibiting HDAC-4 activity in a cell, comprising contacting the cell with a specific inhibitor of HDAC-4, whereby HDAC-4 activity is inhibited.

5

59. The method according to claim 15, wherein the cell is contacted with a specific inhibitor of HDAC-4 activity selected from the group consisting of:

- (a) an antisense oligonucleotide complementary to a region of  
 10 RNA that encodes a portion of HDAC-4, and
- (b) a small molecule inhibitor of HDAC-4.

60. The method according to claim 16, wherein the specific inhibitor is an antisense oligonucleotide complementary to a region of RNA that encodes a

portion of HDAC-4.

61. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is a chimeric oligonucleotide.

5

62. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is a hybrid oligonucleotide.

63. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that has a nucleotide sequence length of from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

10

64. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that has a nucleotide sequence length of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

15

65. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that has a nucleotide sequence length of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

20

66. The method according to claim 17, wherein the cell is contacted with an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11.

25

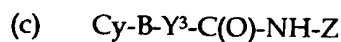
67. The method according to claim 16 wherein the small molecule inhibitor of HDAC-4 has a structure selected from the group consisting of:



5 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S;  
10 S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted;



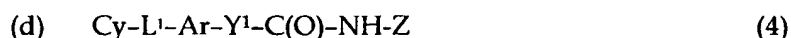
15 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S;  
20 S(O); or S(O)<sub>2</sub>; and Z is aniliny or pyridyl, or thiadiazolyl, any of which may be optionally substituted;



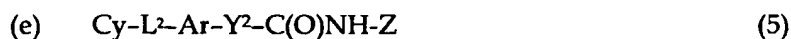
(3)

25 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; B is selected from the group consisting of -CH(OMe), ketone and methylene; Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said

alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted;

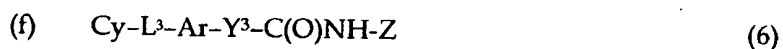


wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, wherein said alkylene may be optionally substituted; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when L<sup>1</sup> is -C(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>-, n being 1, 2, or 3, and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indoliny;



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a

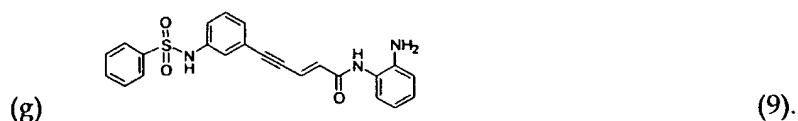
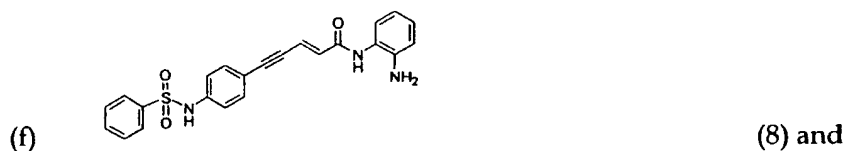
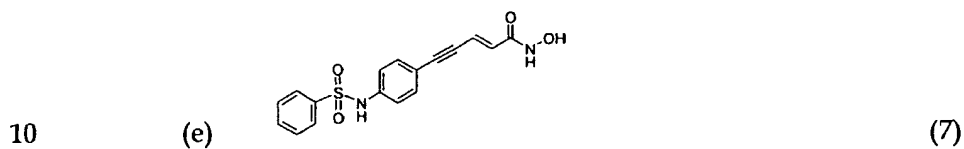
(spirocycloalkyl)heterocyclyl; L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected from the group consisting of O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>2</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, which may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula -C(O)R wherein R comprises an α-amino acyl moiety; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when the carbon atom to which Cy is attached is oxo substituted, then Cy and Z are not both pyridyl;



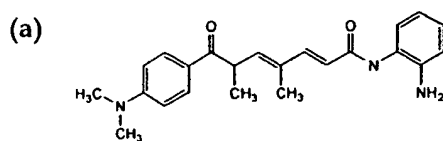
wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>3</sup> is selected from the group consisting of (a) -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; and (b) C<sub>1</sub>-C<sub>6</sub> alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>3</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted

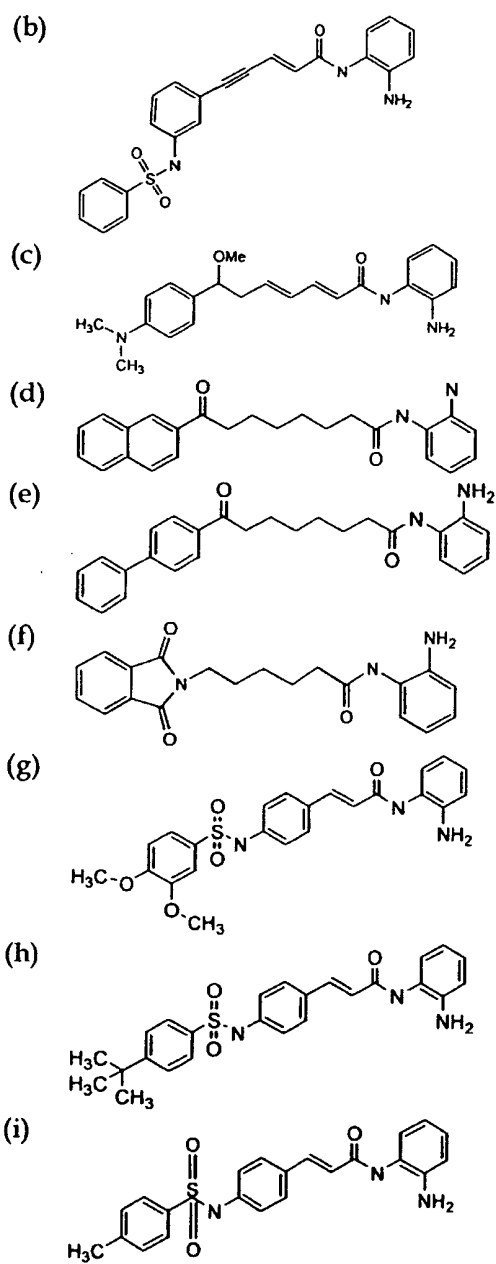


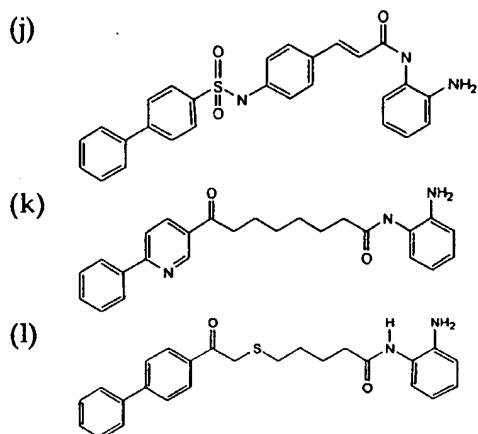
- and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>3</sup> is C<sub>2</sub> alkenylene or C<sub>2</sub> alkynylene, wherein one or both carbon atoms of the alkenylene optionally may be substituted with alkyl, aryl, alkaryl, or aralkyl; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when Cy is unsubstituted phenyl, Ar is not phenyl wherein L<sup>3</sup> and Y<sup>3</sup> are oriented *ortho* or *meta* to each other;



- 15 68. The method according to claim 67, wherein the small molecule inhibitor is selected from the group consisting of:







69. The method according to claim 15, wherein inhibition of HDAC-4 activity in the contacted cell further leads to an inhibition of cell proliferation in the contacted cell.

5

70. The method according to claim 15, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth retardation of the contacted cell.

10

71. A method according to claim 15, wherein inhibition of HDAC-4 activity in the contacted cell further leads to growth arrest of the contacted cell.

15 72. The method according to claim 15, wherein the inhibition of DHAC-4 activity in the contacted cell further leads to programmed cell death of the contacted cell.

73. The method according to claim 26, wherein inhibition of HDAC-4 activity in the contacted cell further leads to necrotic cell death of the contacted cell.

5

74. A method for inhibiting neoplastic cell proliferation in an animal, comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of at least one specific inhibitor of HDAC-4, whereby neoplastic cell proliferation is inhibited in the animal.

10

75. The method according to claim 31, wherein the animal is administered a specific inhibitor of HDAC-4 selected from the group consisting of:

- 15           (a) an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4, and
- (b) a small molecule inhibitor.

76. The method according to claim 32, wherein the animal is administered a therapeutically effective amount of an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-4, whereby neoplastic cell proliferation is inhibited in the animal.

20

77. The method according to claim 33, wherein the animal is administered a chimeric HDAC-4 antisense oligonucleotide.

25

78. The method according to claim 33, wherein the animal is administered a hybrid HDAC-4 antisense oligonucleotide.

5 79. The method according to claim 33, wherein the animal is administered an HDAC-4 antisense oligonucleotide having a nucleotide sequence of from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SED IS NO:4.

80. The method according to claim 32, wherein the animal is  
10 administered an HDAC-4 antisense oligonucleotide having a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SED IS NO:4.

81. The method according to claim 32, wherein the cell is contacted with  
15 an HDAC-4 antisense oligonucleotide that has a nucleotide sequence length of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:4.

82. The method according to claim 32, wherein the animal is  
20 administered an HDAC-4 antisense oligonucleotide that is SEQ ID NO:11.

83. The method according to claim 32, wherein a specific inhibitor is a small molecule inhibitor of HDAC-4 having a structure selected from the group consisting of:

25 (a)  $\text{Cy-CH(OMe)-Y}^1\text{-C(O)-NH-Z}$  (1)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>1</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected  
5 from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the aniliny or pyridyl or thiadiazolyl may be optionally substituted;

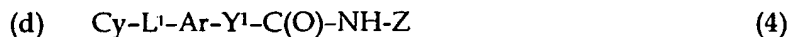
10 (b) Cy-Y<sup>2</sup>-C(O)-NH-Z (2)

wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; Y<sup>2</sup> is C<sub>5</sub> - C<sub>7</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected  
15 from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is aniliny or pyridyl, or thiadiazolyl, any of which may be optionally substituted;

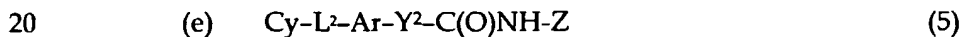
(c) Cy-B-Y<sup>3</sup>-C(O)-NH-Z  
(3)

20 wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; B is selected from the group consisting of -CH(OMe), ketone and methylene; Y<sup>3</sup> is a C<sub>4</sub> - C<sub>6</sub> alkylene, wherein said alkylene may be optionally substituted and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety selected  
25 from the group consisting of O; NR<sup>1</sup>, R<sup>1</sup> being alkyl, acyl or hydrogen; S; S(O); or S(O)<sub>2</sub>; and Z is selected from the group consisting of aniliny,

pyridyl, thiadiazolyl and -O-M, M being H or a pharmaceutically acceptable cation, wherein the anilinyll or pyridyl or thiadiazolyl may be optionally substituted;

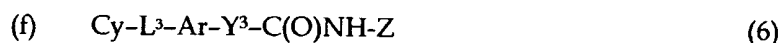


5        wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted; L<sup>1</sup> is -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be  
10       fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; Y<sup>1</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, wherein said alkylene may be optionally substituted; and Z is selected from the group consisting of anilinyll, pyridyl, thiadiazolyl, and -O-M, M being H  
15       or a pharmaceutically acceptable cation; provided that when L<sup>1</sup> is -C(O)NH-, Y<sup>1</sup> is -(CH<sub>2</sub>)<sub>n</sub>-, n being 1, 2, or 3, and Z is -O-M, then Cy is not aminophenyl, dimethylaminophenyl, or hydroxyphenyl; and further provided that when L<sup>1</sup> is -C(O)NH- and Z is pyridyl, then Cy is not substituted indolinyll;



       wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>2</sup> is C<sub>1</sub>-C<sub>6</sub> saturated alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be  
25       substituted, provided that L<sup>2</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by a heteroatom moiety

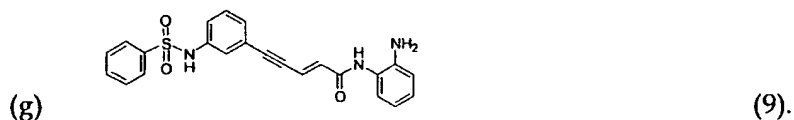
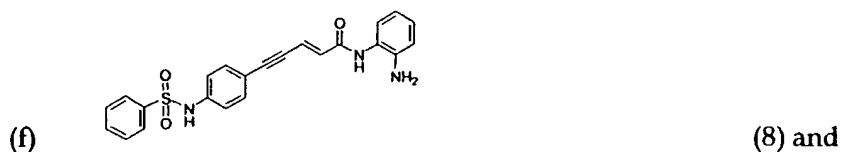
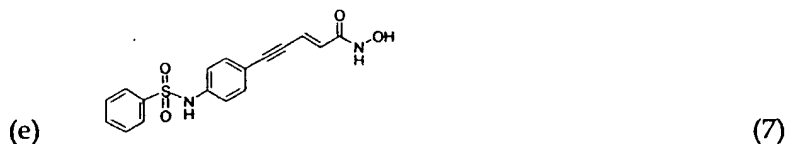
selected from the group consisting of O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>2</sup> is a chemical bond or a straight- or branched-chain saturated alkylene, which may be optionally substituted, provided that the alkylene is not substituted with a substituent of the formula -C(O)R wherein R comprises an α-amino acyl moiety; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when the carbon atom to which Cy is attached is oxo substituted, then Cy and Z are not both pyridyl;



wherein Cy is cycloalkyl, aryl, heteroaryl, or heterocyclyl, any of which may be optionally substituted, provided that Cy is not a (spirocycloalkyl)heterocyclyl; L<sup>3</sup> is selected from the group consisting of (a) -(CH<sub>2</sub>)<sub>m</sub>-W-, where m is 0, 1, 2, 3, or 4, and W is selected from the group consisting of -C(O)NH-, -S(O)<sub>2</sub>NH-, -NHC(O)-, -NHS(O)<sub>2</sub>-, and -NH-C(O)-NH-; and (b) C<sub>1</sub>-C<sub>6</sub> alkylene or C<sub>2</sub>-C<sub>6</sub> alkenylene, wherein the alkylene or alkenylene optionally may be substituted, provided that L<sup>3</sup> is not -C(O)-, and wherein one of the carbon atoms of the alkylene optionally may be replaced by O; NR', R' being alkyl, acyl, or hydrogen; S; S(O); or S(O)<sub>2</sub>; Ar is arylene, wherein said arylene optionally may be additionally substituted and optionally may be fused to an aryl or heteroaryl ring, or to a saturated or partially unsaturated cycloalkyl or heterocyclic ring, any of which may be optionally substituted; and Y<sup>3</sup> is C<sub>2</sub> alkenylene or C<sub>2</sub> alkynylene, wherein one or both carbon atoms of the alkenylene optionally

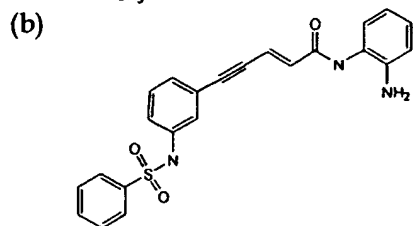
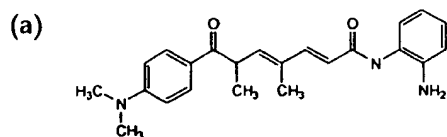


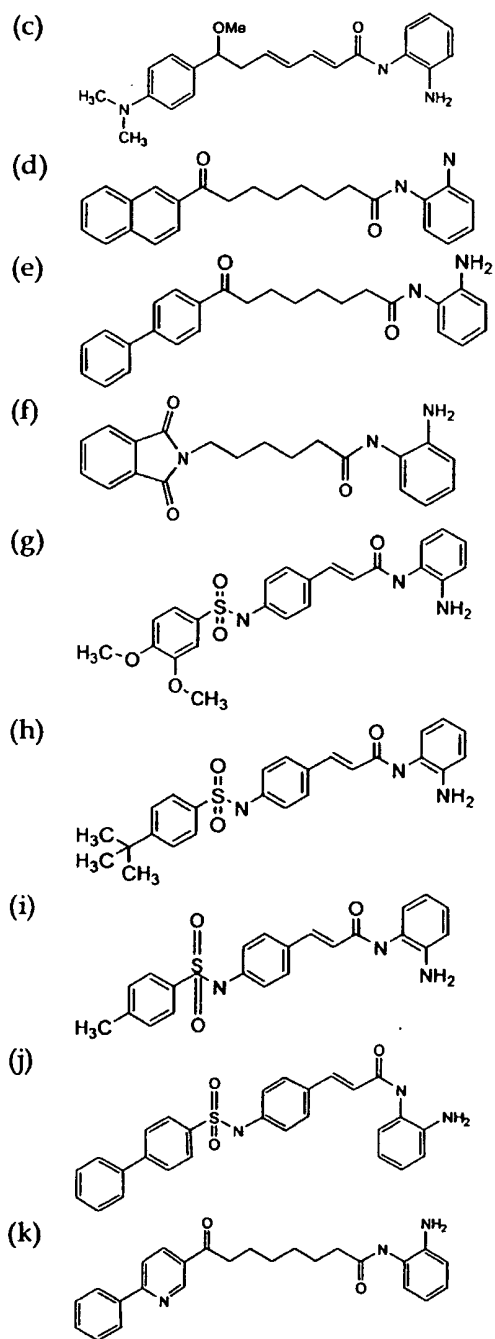
may be substituted with alkyl, aryl, alkaryl, or aralkyl; and Z is selected from the group consisting of aniliny, pyridyl, thiadiazolyl, and -O-M, M being H or a pharmaceutically acceptable cation; provided that when Cy is unsubstituted phenyl, Ar is not phenyl wherein L<sup>3</sup> and Y<sup>3</sup> are oriented *ortho* or *meta* to each other;

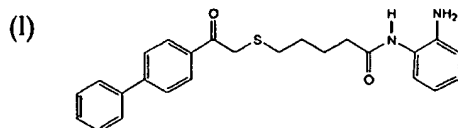


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84. The method according to claim 40, wherein the small molecule inhibitor is selected from the group consisting of:







85. The method according to claim 32, further comprising administering to an animal a therapeutically effective amount of an antisense oligonucleotide complementary to a region of RNA that encodes a portion of HDAC-1.

86. The method according to claim 42, wherein the animal is administered a chimeric HDAC-1 antisense oligonucleotide.

87. The method according to claim 42, wherein the animal is administered a hybrid HDAC-1 antisense oligonucleotide.

88. The method according to claim 42, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence from about 13 to about 35 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.

89. The method according to claim 42, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide sequence of from about 15 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.

90. The method according to claim 42, wherein the animal is administered an HDAC-1 antisense oligonucleotide having a nucleotide

sequence of from about 20 to about 26 nucleotides which is selected from the nucleotide sequence of SEQ ID NO:2.

91. The method according to claim 42, wherein the animal is
- 5 administered an HDAC-1 antisense oligonucleotide that is SEQ ID NO:5.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 September 2002 (12.09.2002)

PCT

(10) International Publication Number  
**WO 02/069947 A3**

(51) International Patent Classification<sup>7</sup>: **A61K 48/00**,  
C12N 15/11, A61K 31/165, C07D 295/14, C07C 225/22,  
225/24, 235/38, 235/78, 237/10, 237/20, 251/48, 259/06,  
271/22, 271/28, 275/34, 275/40, C07D 295/20

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(21) International Application Number: PCT/IB02/02002

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(22) International Filing Date: 14 January 2002 (14.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/261,674 12 January 2001 (12.01.2001) US  
60/261,522 12 January 2001 (12.01.2001) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

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**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

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(88) Date of publication of the international search report:  
9 October 2003

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/069947 A3

(54) Title: ANTISENSE OLIGONUCLEOTIDES AND TRICHOSTATIN ANALOGUE HISTONE DEACETYLASE-4 INHIBITORS AGAINST CANCER

(57) Abstract: This invention relates to the inhibition of histone deacetylase (HDAC) expression and enzymatic activity. The invention provides methods and reagents for inhibiting HDAC-4 and HDAC-1 by inhibiting expression at the nucleic acid level or inhibiting enzymatic activity at the protein level. Specific inhibitors are antisense oligonucleotides against HDAC-4 and HDAC-1 and trichostatin analogues.

## INTERNATIONAL SEARCH REPORT

Intern Application No  
PCT/IB 02/02002

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K48/00 C12N15/11 A61K31/165 C07D295/14 C07C225/22  
C07C225/24 C07C235/38 C07C235/78 C07C237/10 C07C237/20  
C07C251/48 C07C259/06 C07C271/22 C07C271/28 C07C275/34

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K C12N C07D C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 00 71703 A (METHYLGENE INC) 30 November 2000 (2000-11-30)</p> <p>page 9, line 22 -page 10, line 8 page 11, line 14 -page 12, line 15 page 14, line 23 -page 15, line 19 page 25, line 1 - line 9 example 5 claims 1,2,4,8-11,13,14,16,18-21 --- -/--</p>	<p>1-11, 18-29, 44-54, 58-66, 69-82</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- \*Z\* document member of the same patent family

Date of the actual completion of the international search

27 February 2003

Date of mailing of the international search report

28. 07. 2003

Name and mailing address of the ISA

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Authorized officer

Hars, J

Intern:                      Application No  
PCT/IB 02/02002

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C07C275/40 C07D295/20

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 00 23112 A (BESTERMAN JEFFREY M ;MACLEOD ALAN ROBERT (CA); METHYLGENE INC (CA)) 27 April 2000 (2000-04-27)</p> <p>page 8, line 25 -page 9, line 15 page 19, line 1 -page 20, line 25 page 28, line 6 -page 29, line 15 examples 9,10 claims 1,3,7,8,11-13,38,42,43,46-48</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-11, 18-29, 44-54, 58-66, 69-82</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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Authorized officer

Hars, J

## INTERNATIONAL SEARCH REPORT

Intern .pplication No

PCT/IB 02/02002

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 97 35990 A (JAMISON TIMOTHY F ;HARVARD COLLEGE (US); TAUNTON JACK (US); HASSIG) 2 October 1997 (1997-10-02)</p> <p>page 2, line 15 - line 33 page 5, line 8 - line 28 page 6, line 15 - line 29 page 11, line 5 - line 7 page 13, line 1 - line 4 page 27, line 13 - line 34 page 30, line 1 - line 9 page 72, line 34 -page 73, line 4 page 82, line 14 - line 22 figures 6,7 claims 77-79</p> <p>---</p>	<p>1-11, 18-29, 44-54, 58-66, 69-82</p>
A	<p>GROZINGER CHRISTINA M ET AL: "Three proteins define a class of human histone deacetylases related to yeast Hda1p" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 96, no. 9, 27 April 1999 (1999-04-27), pages 4868-4873, XP002159644 ISSN: 0027-8424 abstract</p> <p>---</p>	<p>1-11, 18-29, 44-54, 58-66, 69-82</p>
A	<p>MANFRED JUNG ET AL: "Amide Analogues of Trichostatin A as Inhibitors of Histone Deacetylase and Inducers of Terminal Cell Differentiation" JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. WASHINGTON, US, vol. 42, no. 22, 4 November 1999 (1999-11-04), pages 4669-4679, XP002144226 ISSN: 0022-2623 the whole document</p> <p>---</p>	<p>1-11, 18-29, 44-54, 58-66, 69-82</p>
P,X	<p>DATABASE EMBL_PAT 'Online! EBI27 September 2001 (2001-09-27) METHYLGENE INC.: "Sequence 4 from Patent WO0170675" retrieved from WWW.EBI.AC.UK Database accession no. AX456074 XP002232928 abstract</p> <p>---</p> <p>--- -/--</p>	<p>44,48-50</p>



## INTERNATIONAL SEARCH REPORT

Internu      pplication No  
PCT/1B 02/02002

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>DATABASE EMBL_PAT 'Online! EBI27 September 2001 (2001-09-27) METHYLGENE INC.: "Sequence 11 from Patent W00170675" retrieved from WWW.EBI.AC.UK Database accession no. AX456081 XP002232929 abstract</p>	44,51

# INTERNATIONAL SEARCH REPORT

In                      national application No.  
PCT/IB 02/02002

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:                      —  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.:                      —  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:                      —  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-11, 18-29, 45-54, 60-66, 76-82(entirely); 36, 44, 58, 59, 69-75

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.1

Although claims 1-11,18-29,36,58-66,69-82 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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## Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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## Continuation of Box I.2

Present claims 44,58,69-74 relate to a composition or the use of a composition, defined by reference to a desirable characteristic or property, namely the inhibition of HDAC-4.

The claims cover all composition having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products/compounds/methods/apparatus. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product/compound/method/apparatus by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the antisense oligonucleotides to HDAC-4 as mentioned in claims 1-11,18-29,45-54,59-66,75-82.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## INTERNATIONAL SEARCH REPORT

Inten - Application No

PCT/IB 02/02002

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		CA 2366408 A	30-11-2000
		EP 1173562 A	23-01-2002
		JP 2003500052 T	07-01-2003
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		EP 1243289 A	25-09-2002
		EP 1243290 A	25-09-2002
		EP 1123111 A	16-08-2001
		JP 2002528391 T	03-09-2002
		US 2003096777 A	22-05-2003
WO 9735990 A	02-10-1997	AU 2990597 A	17-10-1997